High performance liquid chromatography of non-steroidal anti-inflammatory agents.

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHY OF NON-STEROIDAL ANTI-INFLAMMATORY AGENTS

submitted by

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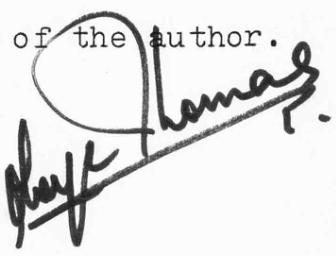
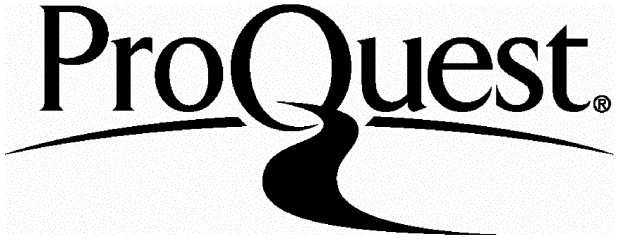
for the degree of Ph.D.
at the University of Bath, 1979.

The research has been carried out in the School of Pharmacy and Pharmacology of the University of Bath under the joint supervision of Professor R.T. Parfitt and Dr. T.M. Jefferies.





SUMMARY

Acidic non-steroidal anti-inflammatory agents represent a large group of closely related compounds prescribed for the many patients suffering from rheumatic diseases. The mode of action of this group of drugs has been related to prostaglandin synthetase inhibition. This action is non-specific and may also be responsible for the gastric irritation and bleeding which constitute a major side effect. It is important therefore for patients being treated with such non-specific high dosing agents to be far better controlled than they are at present. Methods providing simple, rapid analysis of body fluids must be developed and adapted for routine use in clinical situations. These techniques are also necessary for an investigation into possible reasons for disparate drug activities observed in patients with rheumatic disease responding differently to different acidic drugs.

The technique of High Pressure Liquid Chromatography (HPLC) has been explored as a means to these ends. The novel procedure of 'ionic suppression' in HPLC employing a reversed phase column and aqueous acidic solvent, based upon the physico-chemical properties of the acidic anti-inflammatory agents studied, has been developed for routine use. The procedure has been found to afford

flexibility, selectivity, load capacity, sensitivity, speed and convenience over previously reported analytical methods. It is also capable of being exploited in the analysis of newly developed related drugs.

The results obtained from the application of the HPLC procedure developed have been:-

(1) the determination of ten non-steroidal anti-inflammatory agents in plasma and urine,

(2) the application in a clinical trial of Benoxaprofen,

(3) the assistance to clinicians at the Royal National Hospital for Rheumatic Diseases in providing rapid evaluation of patient compliance,

(4) the simultaneous determination of four non-steroidal anti-inflammatory agents and their metabolites in plasma and urine,

(5) the profile study of levels of Sulindac and its metabolites in the plasma of a healthy volunteer after a single oral dose over 24 hours,

(6) the examination of clinical urine and plasma samples of a patient treated with Clinoril and naproxen,

(7) the separation of the glucuronide conjugates of ketoprofen and benoxaprofen,

(8) the detection of hydroxyketoprofen as a metabolite of ketoprofen in rabbit.

HPLC techniques have thus been established by this study as a most valuable aid to the modern therapy of rheumatic diseases. Improvements in detector technology will afford greater sensitivity and should extend the scope of its therapeutic applications.

ACKNOWLEDGEMENTS

I am highly indebted to Professor R.T. Parfitt, the Head of the School of Pharmacy and Pharmacology, and Professor of Pharmaceutical and Medicinal Chemistry for suggesting the topic and also providing facilities for this research. My sincere appreciation goes to him and also to my supervisor, Dr. T.M. Jefferies, whose keen interest, guidance and constant encouragement enabled this work to be completed.

I wish to express my thanks to the Lagos State Government of Nigeria for the award of the research scholarship, and also to Dr. J. Davies and Dr. N.D. Hall of the Royal National Hospital for Rheumatic Diseases for clinical samples of urine and plasma.

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My gratitude is also due to my fiancée for her constant understanding and support.

Humbly dedicated to every member of my family
for their love and unflinching support;
especially to my mother and to the blessed
memory of my father.

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INTRODUCTION.

CHAPTER 1

Non-Steroidal Anti-Inflammatory Agents

Acidic non-steroidal anti-inflammatory agents represent a large group of closely related compounds prescribed for the many patients suffering from rheumatic diseases. These drugs possess both anti-inflammatory and analgesic properties, and also exhibit a range of side effects. Rheumatic diseases may have a minor or major effect upon an individual's health and treatment is largely an attempt to subdue inflammation, limit deformity and relieve pain. The evaluation of the available drugs in order to offer the patient the most efficacious treatment is a continuous process generally carried out by the patient, the general practitioner and, in severe cases, by the consultant rheumatologist under hospital conditions. It is here that recently introduced drugs are evaluated and compared with existing therapy.

Plasma and urine levels of therapeutic agents and sometimes their metabolites may be related to clinical efficacy. Determination of such levels in hospitals is not yet a routine procedure, and for high dosing drugs such as non-steroidal anti-inflammatory agents, this could lead to poor disease control and harmful side effects.

The most obvious biological feature of acidic non-steroidal anti-inflammatory agents is their lack of specificity of action. Their broad spectrum of biological responses, many of which may be unrelated to their desirable response, accounts largely for their non-specific clinical efficacy and for some side effects. Most acidic agents, for example, inhibit a number of proteolytic enzymes including collagenases (1), they uncouple oxidative phosphorylation (2), bind strongly to protein and displace endogenous substances from serum protein binding sites (3). They inhibit the biosynthesis of mucopolysaccharide and act as strong inhibitors of prostaglandin synthetases (4, 5). If this mode of action is non-specific, then side effects, especially gastric irritation due to a decrease in prostaglandin synthesis in the lining of the gut would lead to gastric hyperacidity.

Clearly, it is important for patients being treated with these non-specific, high dosing agents to be far better controlled. As for such control, methods providing simple, rapid analysis of body fluids must be developed and adapted for routine use in clinical situations.

Based upon the structure of indomethacin, a powerful anti-inflammatory agent, a whole range of aromatic acetic acid derivatives has been developed. Furthermore, patients with rheumatic disease appear to respond differently

to different acidic drugs. Hence, the development of appropriate analytical techniques is necessary for an investigation of possible reasons for disparate drug activities.

1.1 Pharmacological Actions.

The major parameter in the design of new anti-rheumatic agents acting via new mechanisms is the definition of a meaningful animal or *in vitro* disease model. All available biological test systems are likely to identify those agents acting by the mechanisms of existing drugs. Most of these agents combat a part of the normal inflammatory response and are best described therefore as anti-inflammatory agents. Such drugs treat symptoms only, that is, relief of pain, maintenance of function and prevention of progressive anatomical deformity

Inhibition of prostaglandin is now considered to be the most important mode of action of these drugs. Vane (4) in 1971 first proposed that anti-inflammatory drugs, including aspirin, operate by inhibiting prostaglandin synthesis. It was also revealed that:-

(i) Prostaglandins were likely to be important in all body functions that are modified by aspirin-like drugs.

(ii) Intermediate prostaglandin synthesis may also have important functions.

(iii) Prostaglandins are the body's natural defences against all forms of change, including those induced by mechanical, pathological, chemical and physiological stimuli. Although inflammation is a defence mechanism, it can become pathological. Prostaglandins are known to induce inflammation in rats and man.

(iv) Finally, the concentrations of unmetabolised aspirin-like drugs found in blood plasma following clinical doses were similar to the concentrations needed in vitro to inhibit prostaglandin synthesis.

Crook and Collins (6) measured prostaglandin synthetase activity in vitro in the microsomal fraction of synovial tissues from rheumatoid arthritic patients. The enzyme was inhibited in vitro by low concentrations of several 'aspirin-like' drugs, although paracetamol and salicylic acid were virtually inactive. It was also illustrated that many non-steroidal anti-inflammatory drugs were more potent inhibitors of the enzyme system, prostaglandin synthetase, than aspirin.

Many thousands of arylacetic acids have now been synthesised and certain molecular features have emerged for compounds with an optimum therapeutic index. From the list of structures of the drugs studied (figs. 3.1A and 1B), an α-methyl group not only enhances anti-inflammatory activity considerably, but endows the molecule with

an asymmetric carbon atom. This affords two geometric forms of the drug each of which may interact differently with the 'receptor' or 'receptors'. Drug geometry as in many other series of therapeutic agents, appears to be important and dextrorotatory agents (S (+) configuration) appear to possess higher in vivo activity than their mirror image form, eg., especially in the case of naproxen, suggesting that at least some receptors are selective. There is certainly high specificity of the S(+) isomers of arylacetic acids in their inhibition of prostaglandin synthetases.

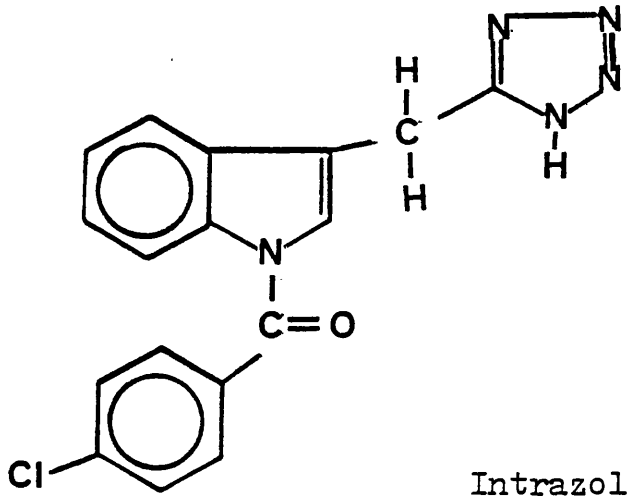
Whatever the modes of action of these drugs, there is a need for the drug to be present in a therapeutic concentration at the site of action. Currently, the concentration in blood is used as a guide to therapy. It is also the only measureable parameter that may indicate the fate of the drug in the patient.

1.2 Side Effects.

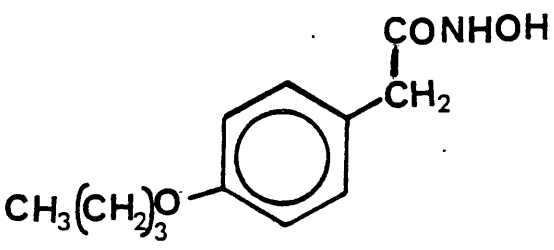
The non-steroidal anti-inflammatory drugs are used to treat chronic conditions and high doses are usually required. A range of undesirable side effects including dyspepsia, skin rashes, gastric irritation and bleeding, and central nervous system toxicity, especially headaches and dizziness, have been well documented by Prescott (7).

The 'acidic' anti-inflammatory agents which include aspirin, arylacetic acids, phenylbutazone and its analogues and the fenamic acids have all been haunted by the spectre of gastric irritation. Attempts to mask the acidity, however, by employing groups other than carboxylic acid groups have met with little success, for example, intrazole employing the acidic tetrazole moiety and bufexamac with a hydroxamic acid function, fig. 1.2.

FIGURE 1.2 Structures of Intrazole and Bufexamac.



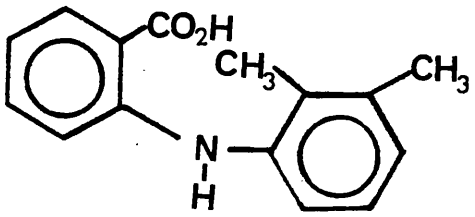
Intrazole



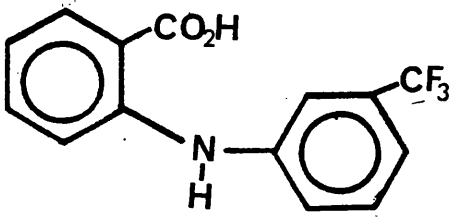
Bufexamac

A number of anthranilic acid anti-inflammatory agents known collectively as fenamates, fig. 1.3, could conceivably be related to aspirin. Gastric irritation together with other toxic manifestations, eg., papillary necrosis in animals, renders the fenamates the least popular group of acidic anti-inflammatory agents.

FIGURE 1.3 Some Fenamates.



Mefenamic Acid



Flufenamic Acid

It is possible that the ability of acidic anti-inflammatory drugs to inhibit non-specific prostaglandin synthetases is the very property that results in gastric irritation, for decreased prostaglandin biosynthesis does lead to gastric hyperacidity and it is likely that local release of prostaglandins plays an important role in gastric secretion control. The claims of greater efficacy and safety made for newer agents marketed cannot be wholly justified unless it is possible to design acidic agents that are selective inhibitors of prostaglandin synthetases.

There is however, a second group of drugs that not only offer symptomatic relief but retard the disease by attacking the causative chronic inflammatory processes. These include D-penicillamine, levamisole, gold compounds, and some anti-malarials such as chloroquine and hydroxychloroquine. These drugs are often slow in their onset of action and are sometimes accompanied by severe side effects. They therefore require careful patient monitoring.

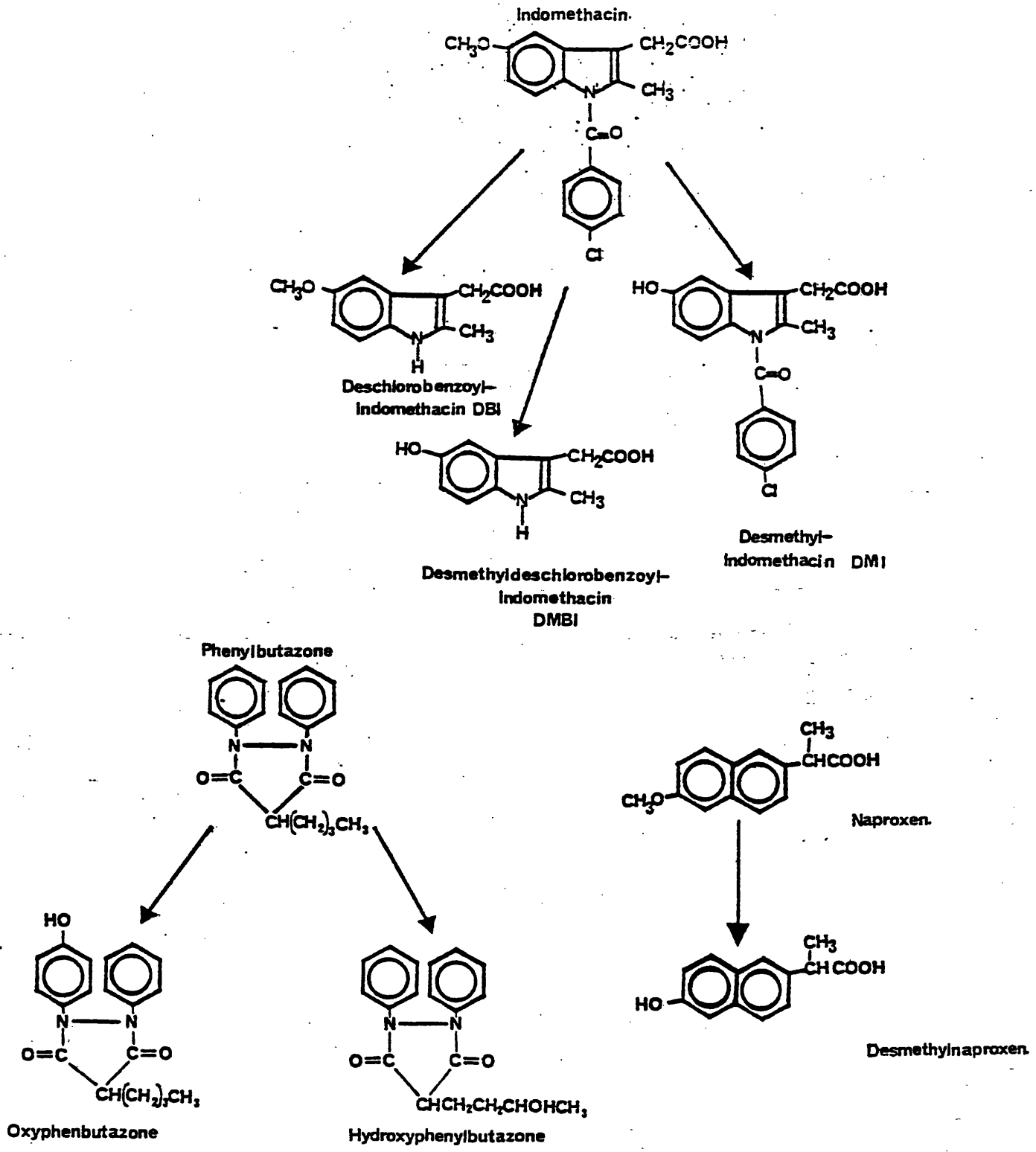
1.3 Excretion.

Most of the non-steroidal anti-inflammatory drugs are excreted as glucuronide conjugates. However a few, including Indomethacin, Naproxen, phenylbutazone and Sulindac undergo metabolism before conjugation as shown in Table 1.1 and fig. 1.4A & B. The primary metabolites of indomethacin and naproxen are inactive. Phenylbutazone is metabolised into oxyphenbutazone and hydroxyphenylbutazone. Oxyphenbutazone is a very potent metabolite and is also administered as a drug alone. The sulphide metabolite of Sulindac is more potent and even has a longer half-life in plasma than sulindac itself. It is not excreted in urine but converted to the sulphone metabolite which is inactive.

TABLE 1.1 Metabolites of Some Non-Steroidal Anti-Inflammatory Agents.

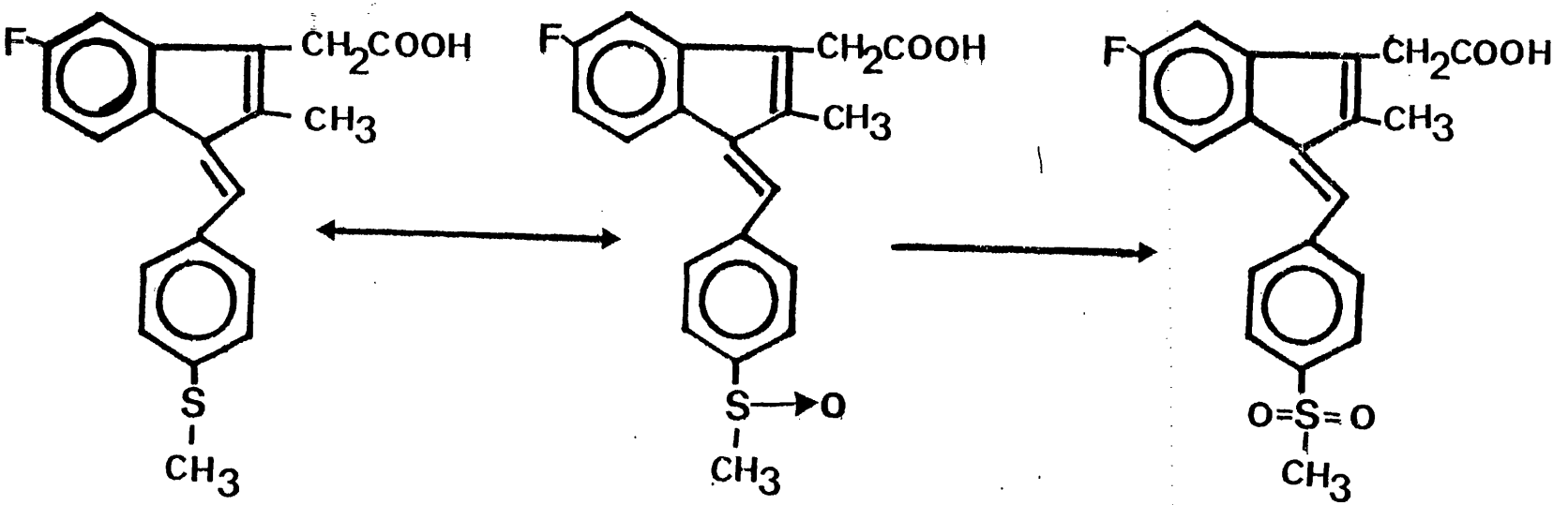
	Drug	Metabolite	Metabolite Activity	Ref
1.	Indomethacin	Deschlorobenzoylindomethacin	Inactive	8
		Desmethylindomethacin	Inactive	8
2.	Naproxen	Desmethylnaproxen	Inactive	9, 10
3.	Phenylbutazone	Oxyphenbutazone	Active	11
		Hydroxyphenbutazone	Inactive	11
4.	Sulindac	Sulphide metabolite	Active	12
		Sulphone metabolite	Inactive	12

FIGURE 1.3A Metabolites of Indomethacin, Phenylbutazone and Naproxen.



Sulindac and its Metabolites.

Fig. 1.3B



Sulphide

Sulindac

(Sulphoxide)

Sulphone

1.4 Methods of Analysis.

Various chromatographic and spectrophotometric methods of analysis have been used to identify and determine these non-steroidal anti-inflammatory agents.

In 1953, Burns et al. (13) applied UV spectrophotometry to the analysis of phenylbutazone extracted into hexane from acidified plasma and urine. The phenylbutazone was removed from the hexane using aqueous alkali and the UV absorbance determined at 265nm. The method was later applied to metabolites of phenylbutazone extracted from biological fluids (11). The UV spectra for ketoprofen, naproxen and fenoprofen were published by Lotti (14) in 1975. Each compound was dissolved in methanol and the absorbances measured between 220 and 320nm. This data provided a method for identification and possible quantitative determination of these agents.

Hucker, et al.(15) measured indomethacin by spectrophotofluorimetry at an excitation maximum of 295 and emission maximum of 385nm.

Harman, et al.(8) investigated paper and thin-layer chromatographic separations of urinary metabolites of indomethacin. The separation of indomethacin, its metabolites and their glucuronide conjugates was obtained on Whatmann 3MM paper with methanol-water-n-butylalcohol-benzene (2:1:1:1) as the solvent. The thin-layer

chromatographic behaviour of naproxen, ketoprofen and fenoprofen had also been reported (14). These methods, though not quantitative could be used for preliminary identification and preparative analysis to obtain pure compounds for further studies.

More accurate, specific and sensitive methods of analyses of these agents were needed for pharmacological and pharmacokinetic studies. Various gas chromatographic precedures were developed for some of these compounds and the conditions used are summarized in Table 1.2.

From this table, it can be seen that:

(i) Derivatization of the free acid was usually necessary, either to the methylester or to the trimethylsilyl ester. The latter would improve the volatility of the compound. Phenylbutazone is not structurally suitable for derivatization, and consequently is less readily detected.

(ii) The stationary phases used are generally non-polar, SE-30, Apiezon-L; to slightly polar, OV-17, XE-60.

(iii) The detection by FID is usually sensitive enough for quantifications in plasma, especially when derivatives were formed. However, detections by ECD used for indomethacin and ^{63}Ni-ECD for ketoprofen greatly improved sensitivity.

TABLE 1.2 Gas Chromatographic Analytical Conditions Reported for Some Non-Steroidal Anti-Inflammatory Agents.

Drugs	Stationary Phase	Support Material	Temp.	Derivative	Detector	Limit Detected	Ref.
Alclofenac	3% XE-60	Supelcoport 80/100 mesh	230°C	TMS	FID	0.25µg	16
Fenoprofen	3.8% UCCW-982	Diatoport	175°C	TMS	FID	0.25µg	17
Ketoprofen	5% OV-17	Chromosorb W 80-100	200°C	Not derivatized	Ni-ECD	40ng	18
Indomethacin	2%(W/W) SE-52	Chromosorb	245°C	Methyl-ester	ECD	50ng	19
Naproxen	3.8% SE-30	Diatoport	200°C	Methyl-ester	FID	0.25µg	20
Phenylbutazone	2.5% SE-30	Gas Chrom Q 100-120	195°C	Not derivatized	FID	1µg	21
	3% Apiezon-L	Chromosorb-W 80-100	230°C		FID	0.5µg	22

In general, sample preparation is a significant part of analyses because of the series of extraction procedures and the need for derivatization. Finally, analysis at such high temperatures might result in decarboxylation of some of the drugs.

The only ion-exchange chromatographic method reported was for naproxen (23). Urine composite aliquots were pipetted onto a 35x1.8 cm.id column charged with 35meq. of ion exchange resin. The biotransformed components related to naproxen were then separated by successive elution with different buffers, over a pH gradient from 3.5 to 6.5. About twenty bio-transformed products of Naproxen were detected in human and animal studies.

The technique of HPLC was introduced for the analysis of these agents in 1974. Pound, et al. (24) described a method for the assay of phenylbutazone in plasma. Phenylbutazone was extracted into n-hexane from acidified plasma and chromatographed on a Sil-X adsorbent column with 10% tetrahydrofuran in n-hexane as the mobile phase. The detection was by a UV-monitor at 254nm and the limit detected was 0.2µg/ml. By using the same stationary phase and a slightly modified mobile phase containing 0.002% acetic acid and 23% THF in n-Hexane, the simultaneous determination of phenylbutazone and its metabolite, oxyphenbutazone was achieved (25). Both procedures,

though more sensitive than the previously reported GC methods (21,22), still involved extraction of the drugs into organic solvents because the stationary phase used was not compatible with aqueous samples.

An attempt to minimize sample preparation led to the development of a reversed phase HPLC procedure to determine indomethacin in a mixture of indomethacin, frusemide and aspirin. Aqueous and slightly acidic acetonitrile (pH 5) was the solvent pumped through a column, (60.93 x 0.21 cm. id), dry-packed with Bondapack C_{18}. Initial extraction of indomethacin from plasma was still necessary with the extract reconstituted in the mobile phase before injection on to the column (26). This procedure has been found to produce a broad peak for indomethacin. The peak was however improved at a lower pH of 3 employed in this work.

CHAPTER 2

The Technique of High Performance Liquid Chromatography

2.1 Perspective

HPLC has become a major analytical technique in the fields of Pharmacy, Biochemistry and Chemistry. Chromatography in its many forms is basically a separation technique. Separation is achieved through a differential migration process whereby the sample components are selectively retained or delayed by the stationary phase. The sample mixture is introduced onto a bed of stationary phase, and swept through it by mobile phase at a rate dependent upon the mutual interactions of sample components with the stationary phase and the mobile phase. Generally, these interactions differ in magnitude for the different sample components so that their rate of passage through the stationary phase bed differs and separation is thus achieved.

Stationary phases largely promote separation of molecules by one or more of the four basic retention mechanisms, namely:-

(i) Adsorption: the ability to physically sorb solutes from solution.,

(ii) Partition: the ability to dissolve solutes when contacted with solutions in an immiscible solvent.

(iii) Ion exchange, the ability to chemically sorb solutes from solution; and

(iv) Gel permeation; a process structure which can retain some, and reject other, solutes on the basis of solute size or shape.

These four retention mechanisms are available to users of HPLC technique, plus a rapidly expanding range of bonded phase materials that include the reversed phase packings. Therefore, the separating potentials of HPLC is probably greater than in any other chromatographic technique.

The different retention mechanisms all have one feature in common, namely that a mixture of solute compounds is separated into individual component bands or peaks, to produce the chromatogram. The interpretation of the chromatogram provides the basis for the utilization of the technique qualitatively and quantitatively.

2.2.1. Retention Parameters.

In a given chromatographic system, the time taken and the volume of mobile phase required to elute a specific solute from the stationary phase bed is constant. Therefore the time or volume needed to elute one substance relative to another particular compound will also be constant. These facts lead to considering two aspects of retention in chromatography:-

(a) Various methods of describing retention and their inter-relations, and

(b) The use of retention as means of the identification of solutes.

Retention parameters can be measured in two ways:-

2.2.1.1 Column Capacity Ratio, K'.

It is a measure of the ratio of the amount of solute in the stationary phase compared to the mobile phase. It is proportional to the distribution coefficient K and the volumes of stationary and mobile phases, Vs and Vm respectively.

$$K' = K. \frac{Vs}{Vm} \qquad \text{(1)}$$

The average fraction of time spent by a molecule in the stationary phase $= \frac{K'}{1+K'}$ (2)

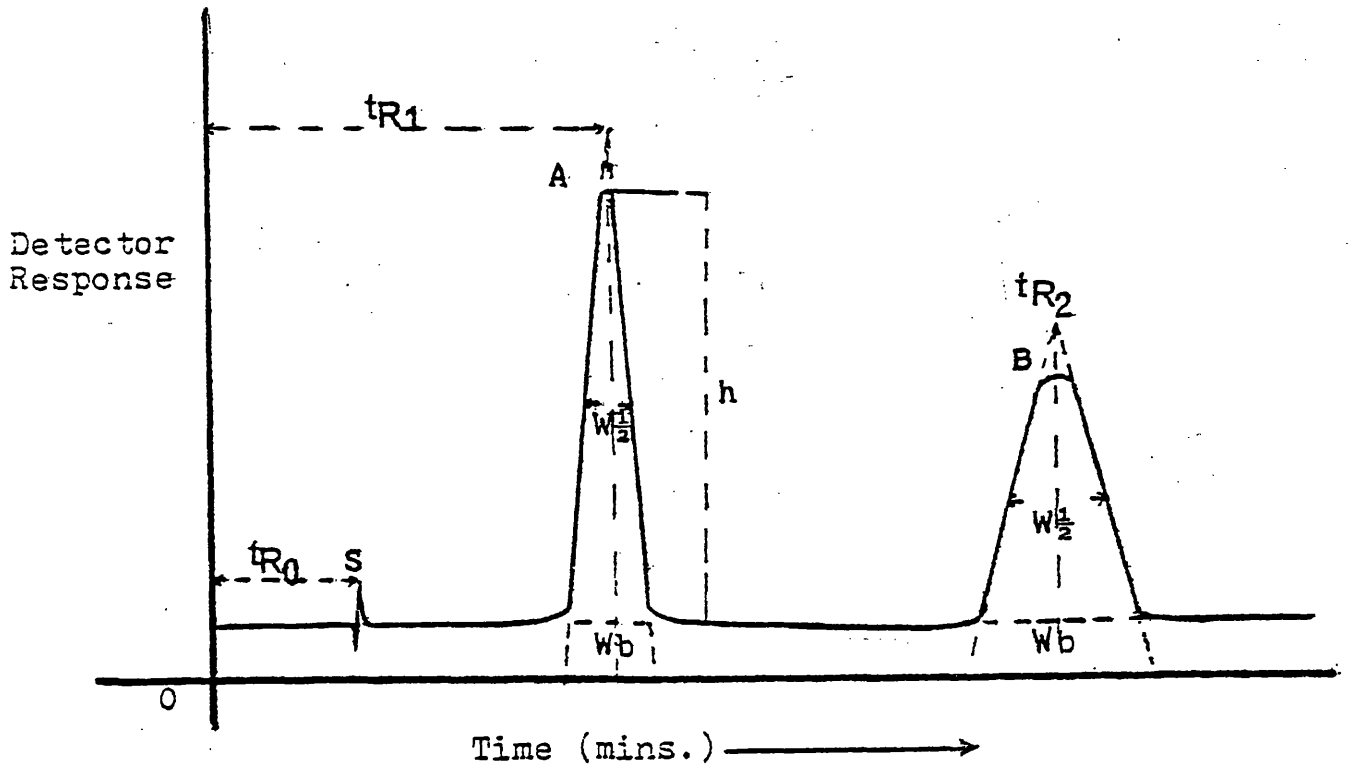
and in the mobile phase $= \frac{1}{1+K'}$ (3)

K' can be expressed as

$$K' = \frac{tR - tRo}{tRo} \qquad (4)$$

tR and tRo are readily obtainable for any solute from the chromatogram, figure 2.1.

FIGURE 2.1



tR = Retention time of the solute; it is the period of time required for the solute to pass from the injector through the column and the detector. It is measured directly on the chromatogram from the point of injection to the apex of the solute peak and is expressed in mins. or mm.

tRo = Retention time (distance) of an unretained compound usually taken as the first peak to emerge. It is generally the solvent in which the solute was dissolved. For reversed phase materials, KNO_3 is suitable.

$$K' = \frac{Vr-Vm}{Vm} \qquad (5)$$

Vr = Retention or elution volume of the solute.

Vm = Total volume of mobile phase in the column including pores, and is given by:-

tRo x f = the volumetric flow rate.

K' is therefore the measure of number of column volumes needed to elute the solute. It can be seen that K' increases by 1 for each 'Column Volume' needed to elute the solute. It is linear with time, volume and chart distance.

2.2.1.2 Retention Ratio, R.

This is the fraction of solute in the mobile phase.

$$R = \frac{1}{1+K'} = \frac{tRo}{tR} = \frac{Vm}{Vr} = \frac{Zo}{Zf} \qquad (6)$$

where Zo = The distance travelled by the solvent,

Zf = The distance travelled by the solute on a column, plate or paper.

Retention on columns is measured by R, while on TLC and paper by Rf, i.e. $\frac{Zo}{Zf}$.

Thus $K' = \frac{1-R}{R}$ (7)

2.2.2 Column Efficiency.

This is the ability of the column to minimise peak spreading. It is expressed quantitatively as the number of Theoretical Plates (N) which is derived from peak width and retention time;

From figure 2.1;

$$N = 16\left[\frac{tR}{Wb}\right]^2 = 5.54\left[\frac{tR}{W\frac{1}{2}}\right]^2 \qquad (8)$$

where $W\frac{1}{2}$ = the peak width at half the peak height and is a measure of the peak shape.

The characteristic of a column is more precisely defined by using the Height Equivalent of a Theoretical Plate (HETP), commonly referred to as Plate Height (H), and is expressed as

$$H(mm) = \frac{\text{Length of the Column (mm)}}{N}$$

H is smaller for (i) better columns,

(ii) small particle diameter,

(iii) less viscous solvents,

(iv) higher temperatures,

(v) small solute molecules,

(vi) slow flow rate.

The lower the value of H - generally between 0.01 and 0.08mm, the better is the column performance.

2.2.3 Band Broadening.

When a small volume of solute is injected sharply into a column, the solute spreads out gradually as it migrates. If the distribution ratio K is independent of concentration, then the concentration profile assumes the Gaussian shape, and the relationship between solute concentration in mobile and stationary phases is linear.

Figure 2.2 shows basic isotherm shapes and their effect on peak shape and on retention time. Reducing solute size improves tailing and fronting. The slope of the graph is the capacity factor, R; and the point D, indicates the limit of linear behaviour for B and C.

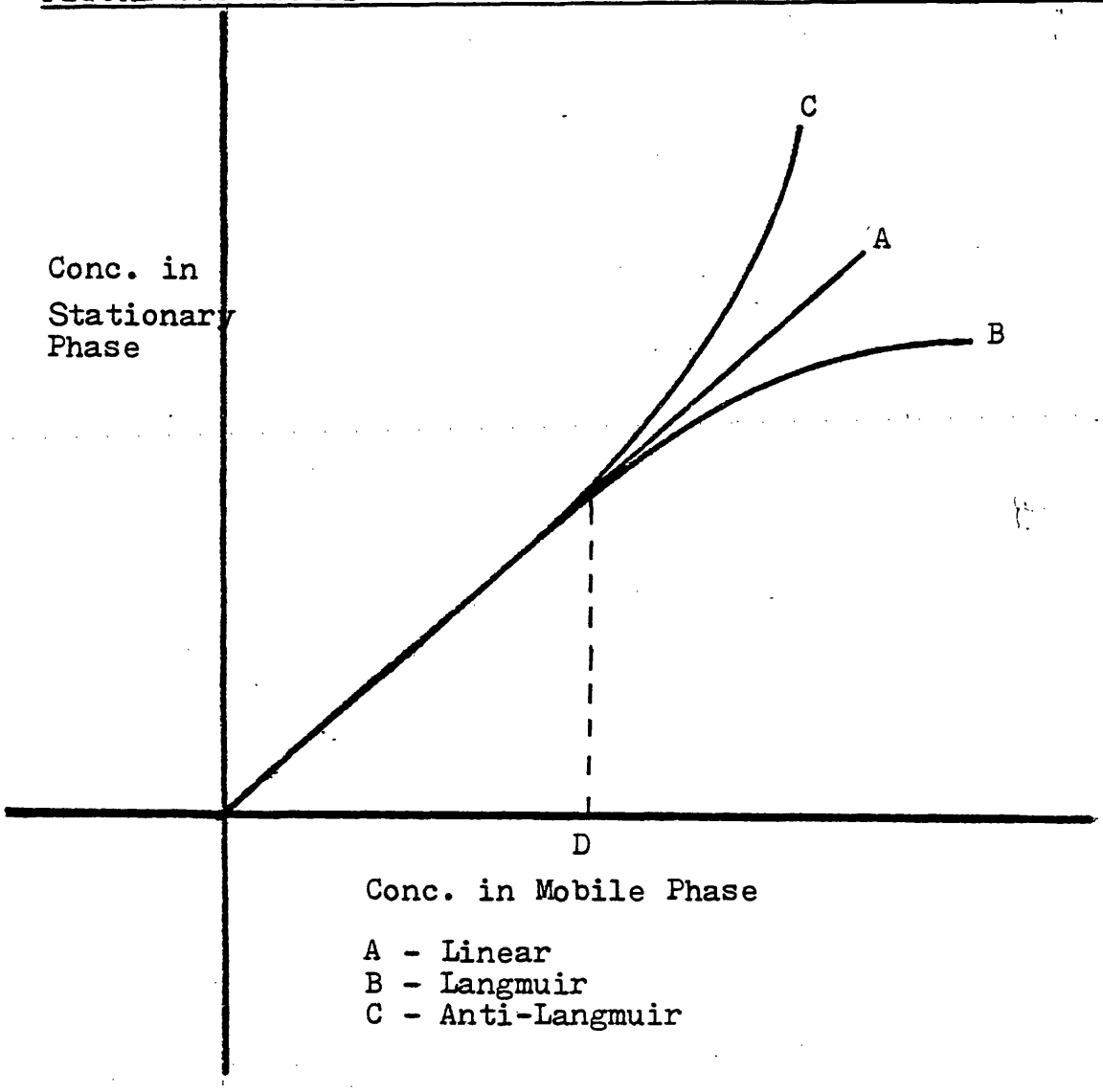
The factors contributing to band-broadening are:-

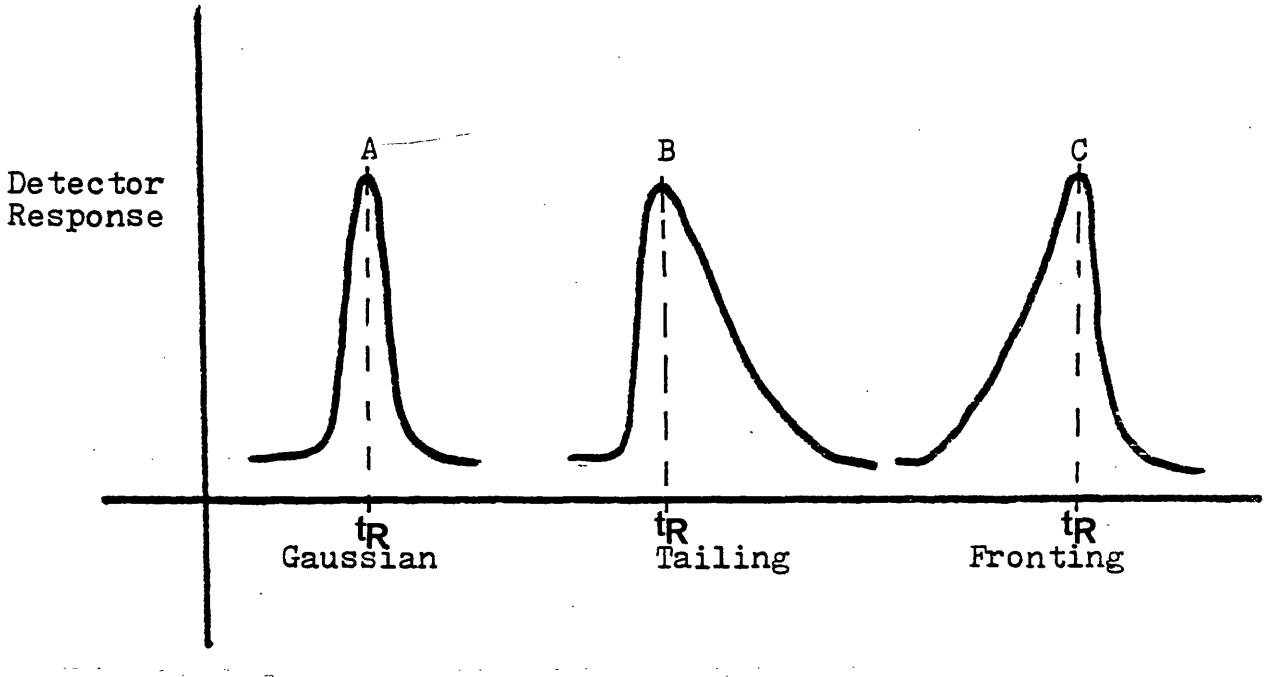
(1) Eddy Diffusion

(2) Molecular Diffusion

(3) Mass Transfer.

FIGURE 2.2 General Characteristics of Sorption Isotherms





The mobile phase could be a gas as in GC or a liquid and the relative order of magnitude of their physical constants are compared in Table 2.1.

TABLE 2.1

Physical Constants	Gas	Liquid
Diffusion Coefficient, Dm, Cm^2S^{-1}	10^{-1}	10^{-5}
Density, ρ, gCm^{-3}	10^{-3}	1
Viscosity, η, $gCm^{-1}S^{-1}$	10^{-4}	10^{-2}
Reynolds' Number	10	100

2.2.3.1 Eddy Diffusion.

The velocity of a solute molecule passing through a packed column will vary greatly according to the flow path in which it happens to be. Additionally, solute molecules may transfer laterally by diffusion and by convection from one flow path to another. This coupling of eddy and lateral diffusions results in a decrease in the amount of band broadening.

2.2.3.2 Longitudinal Molecular Diffusion.

This is a very important factor in increasing band broadening at low flow rates in GC, due to the high value for Dm, whereas in HPLC, it is so small that it can be ignored. The contribution that it makes to the plate height is expressed by

$$\frac{2\gamma Dm}{\mu} \qquad (9)$$

where u = Flow velocity

γ - Factor indicating the degree to which diffusion is obstructed by the column packing. It is <1, for HPLC it is about 0.6.

Dm = Diffusion Coefficient of solute in the mobile phase.

In GC, Dm is reduced at higher flow rates and lower temperatures, hence the effect of temperature upon resolution.

2.2.3.3 Mass Transfer.

This takes place in:-

1. The Stationary Phase:- It is the rate at which solute molecules transfer into and out of the stationary phase. In a liquid stationary phase, the rate of diffusion through the liquid layer controls the rate of mass transfer. Thin layers of low viscosity give higher rates, but solid supports must not be exposed. It is expressed as:-

$$\frac{K'}{(1+K')^2} \cdot \frac{d^2 u}{Ds} \qquad \text{(10)}$$

or

$$Cs \cdot \frac{d^2 u}{Ds} \qquad \text{(11)}$$

where Cs = A constant α to the retention ratio and shape of the column packing.

d = Thickness of liquid stationary phase.

Ds = Diffusion coefficient of solute in the stationary liquid phase.

2. Mobile Phase - Movement of solute molecules in the mobile phase at different speeds due to the different paths followed increases band broadening. Coupling the contribution from eddy diffusion with that from lateral transport diffusion gives the relationship,

$$= \frac{1}{\frac{1}{2\lambda dp} + \frac{Dm}{Cmdp^2 u}} \qquad \text{(12)}$$

where λ = a constant depending on packing structure.

dp = particle diameter.

Cm = a function of the packing structure and column dimension.

The presence of 'stagnant' mobile phase occurring in the less accessible regions of the totally porous stationary phases also increases band broadening. Its contribution to H is measured as

$$\frac{Csm \cdot dp^2 \cdot u}{Dm} \qquad \text{(13)}$$

where Csm is a function dependent upon the fraction of total mobile phase that is considered to be stagnant.

Thus in HPLC, it is very important that the particles are porous, and the pores are uniform with a minimum of poorly accessible areas.

2.2.3.4 Expression for Plate Height, H.

The sum of all these contributory factors provide a value for H, i.e.

$$H = \frac{2\gamma Dm}{u} + \frac{Csd^2u}{Ds} + \frac{1}{\frac{1}{2\lambda dp} + \frac{Dm}{Cmdp^2u}} + \frac{Csmd^2u}{Dm} \quad \text{---(14)}$$

- $\frac{2\gamma Dm}{u}$ → Longitudinal Diffusion
- $\frac{Csd^2u}{Ds}$ → Mass Transfer on Stationary Phase
- $\frac{1}{2\lambda dp}$ → Eddy Diffusion
- $\frac{Dm}{Cmdp^2u}$ → Lateral Mass Transfer
- $\frac{Csmd^2u}{Dm}$ → Mass Transfer 'Stagnant'

Equation 14 is a development of the van Deemter (27) equation developed to consider the factors determining H in GC. Often simplified to

$$H = A + \frac{B}{u} + C.u \qquad \text{[15]}$$

the new equation simplifies to

$$H = \frac{B}{u} + Cs.u + Csm.u + \left[\frac{1}{A} + \frac{1}{Cmu}\right]^{-1} \qquad \text{[16]}$$

Comparing GC and HPLC for H and u (figure 2.3), in GC, the optimum gas flow rate for maximum resolution is when H is at minimum. Flow rates below this give poor H

values due to an increase in longitudinal diffusion. Hence analyses are performed above the minimum flow rate. In HPLC, in practice, low flow rates may be used without increasing H seriously.

The performance of different particle sizes may be compared using the reduced parameters proposed by Knox (28)

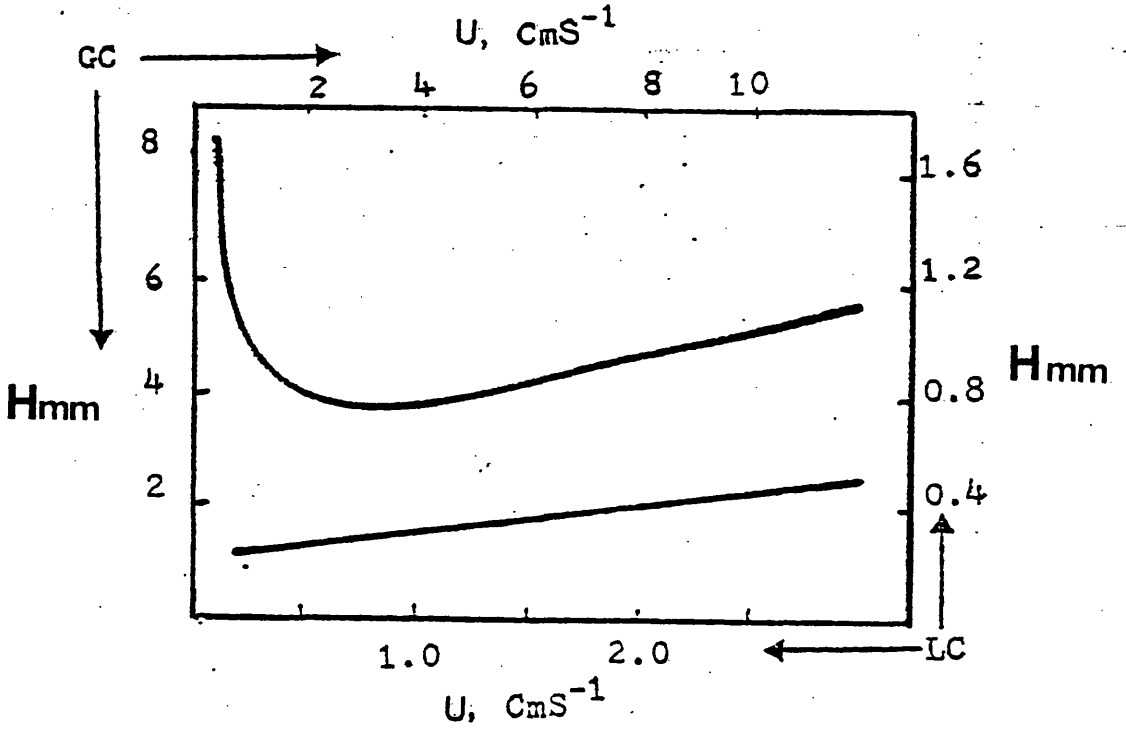
where Reduced Plate Height $h = \frac{H}{dp}$ ——— (17)

and Reduced Velocity $v = \frac{U.dp}{Dm}$ ——— (18)

The general relationship of reduced plate height and reduced velocity may be simplified to

$$h = \frac{B}{V} + Av^{0.33} + Cv \qquad \text{[19]}$$

FIGURE 2.3 Comparison of H Vs U Curves in GC and HPLC.



2.2.3.5 Extrabed Contributions to Band Spreading.

The final concentration profile of the solute in the chromatogram as revealed by the detector also includes the contribution of other factors. These are mainly:

(1) The sharpness of the sample concentration profile at the start of the chromatography due to mode of injection.

(2) Non-linearity of solute distribution between stationary and mobile phase as a function of concentration, and

(3) Change in solute band shape after chromatography due to the connections between the column and detector, and also due to the detector cell volume.

2.2.4 Resolution, Rs.

This is a measure of the separation between two adjacent bands, and is defined as the distance between the two band centres divided by the average band width, figure 2.1.

$$Rs = \frac{tR_2 - tR_1}{\frac{1}{2}[W_1 + W_2]} \qquad (20)$$

when Rs 0.8, < the separation is < 95%.

1.0, ≙ the separation is 98%.

1.5, + the separation is complete.

Resolution may also be written as

$$Rs = \frac{\sqrt{N}}{4}\left[\frac{K'_2 - K'_1}{1 + K_2}\right] \qquad (21)$$

$$= \frac{\sqrt{N}}{4}\left[\frac{\alpha - 1}{\alpha}\right]\left[\frac{K'_2}{1 + K'_2}\right] \qquad (22)$$

where α = Selectivity Factor $\left[\frac{K'_2}{K'_1}\right]$

These are the factors affecting resolution and they are essentially independent so that each may be optimized in turn:-

α - The most important factor and is determined by the combination of the packing material and the eluent system.

K' - Resolution is improved by increasing K', and the effective range is 1-10. It is altered by changing the eluent strength in HPLC, while in GC by altering the temperature.

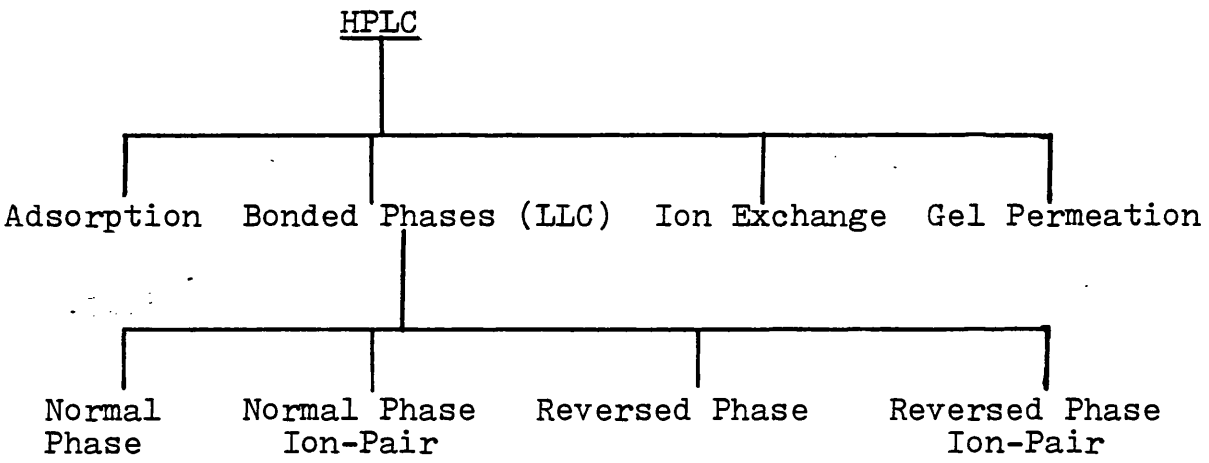
N - Column efficiency expressed as the plate number.

Because $Rs \propto \sqrt{N}$, a large increase in N is needed to significantly improve Rs. Since $N = \frac{L}{H}$, N can be increased by using a longer column, although band spreading will also increase.

2.3 Bonded Phase Liquid-Liquid Chromatography.

Most of the problems associated with classical LLC have been overcome by the development of support materials containing chemically bonded polar or non-polar groups that are unaffected by solvent changes. This is a major advance in the technique of HPLC because of its relevance to the selectivity of the separation process.

FIGURE 2.5 Retention Mechanisms in HPLC.



The bonds are commonly produced by silylation of the free silanol groups that occur over the surface of silica, using reagents devised for GC.

$$\equiv Si\text{-}OH + R_3SiCl \longrightarrow \equiv Si\text{-}O\text{-}SiR_3$$

A Monochloro-Organo Silane.

Methoxy or ethoxy silanes are the most reactive of the silanes. Further reagents are used to extend this

bond to produce chains of the required length and functional groups, figure 2.6. The bond resulting from the silylation reaction is the siloxane bond;

$$\equiv Si - O - Si \equiv$$

which is thesame as occurs within the silica support material. It is stable to mobile phases between pH 2 and 8, but above this range, the silica material will dissolve in the mobile phase.

2.3.1 Reversed Phase Material.

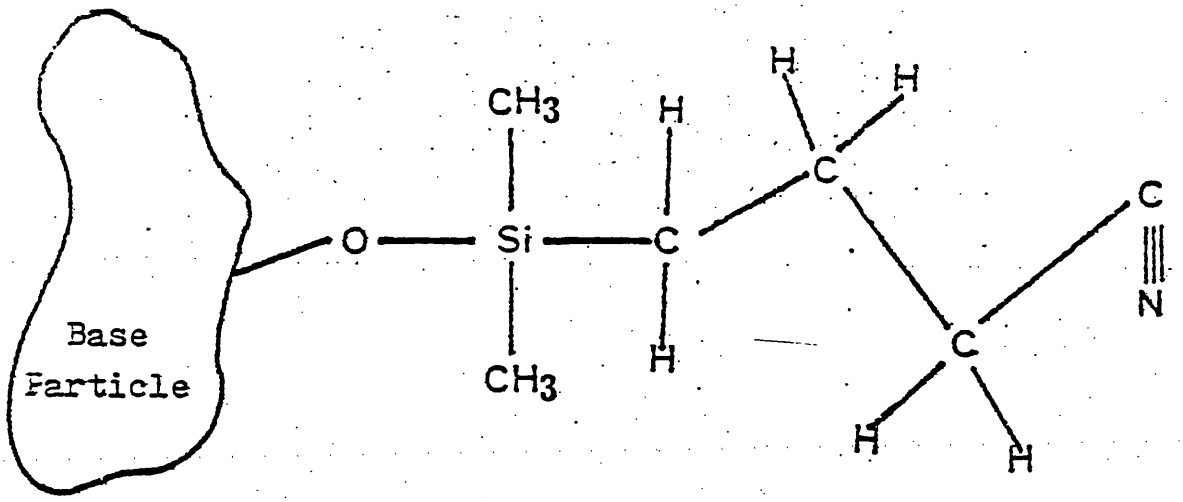
The entire surface of the silica including pores within the particle, is chemically bonded with hydrocarbon chains, figure 2.6B, eg., C2, 6, 8 or 18. OCTADECYL (C18) is the most commonly used, although different commercial forms vary according to:-

(1) Percentage (%) carbon loading;

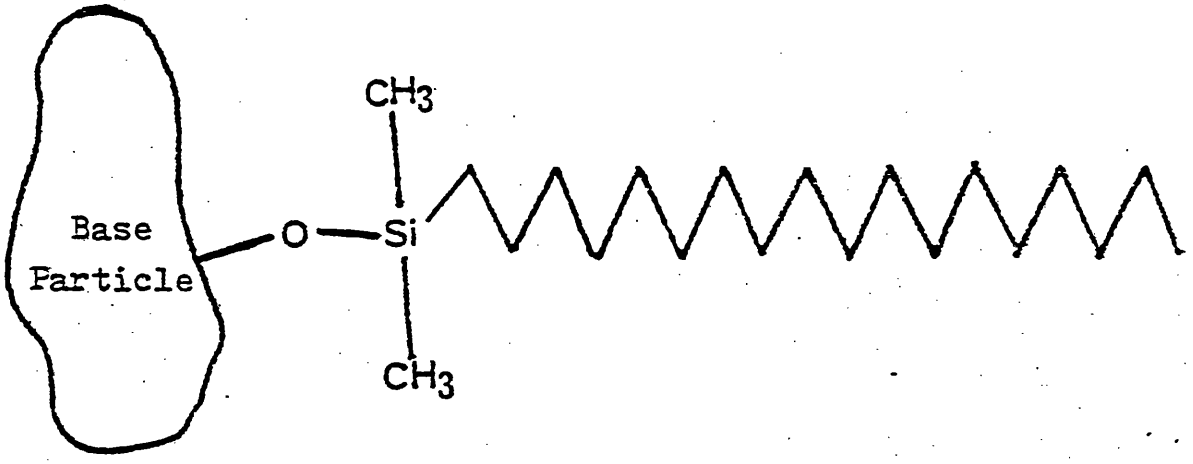
Partisil - 10-ODS	5%
Spherisorb - 5-ODS	7%
Hypersil - 5-ODS	16%
RP-18 Monomeric brush	20%
Partisil - 10-ODS-2 Polymeric brush	20%.

(2) Whether the remaining free silanols are silylated i.e. 'capped' or not, figure 2.7. The extent of capping depends on accessibility to the silylating reagent, and 100% capping is not possible because of steric hindrance

FIGURE 2.6 Bonded Phases.

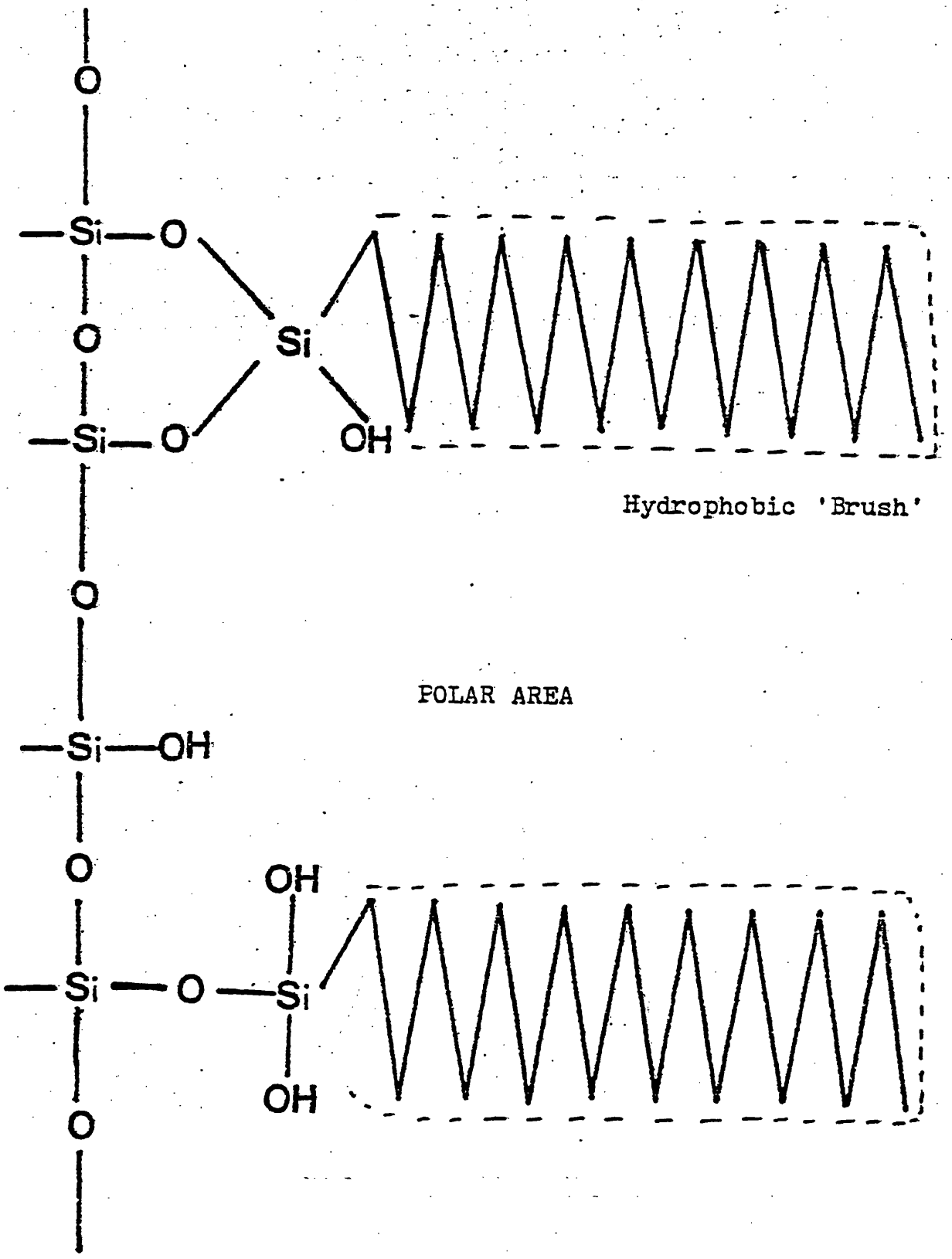


A. Polar Bonded Phase - CN-Stationary Phase.



B. Non-Polar (Reversed Phase) Phase - ODS-C18.

FIGURE 2.7 Surface of Reversed Phase Material.



NOTE: When CAPPED, $-\overset{|}{\underset{|}{Si}}-OH \longrightarrow \overset{|}{\underset{|}{Si}}-O-Si(CH_3)_3$

The slight variations in properties of the various commercial forms of these phases provides further flexibility in the choice of packing material for a particular separation.

2.3.2 Solvent Interaction With the Bonded Phase.

For all reversed phase materials, the surface is predominantly hydrophobic, and is used with an aqueous mobile phase containing an organic modifier, usually methanol or acetonitrile. Under these conditions, the uncapped surface hydroxyls are highly solvated with water and so are deactivated. Hence, a solute molecule must be able to displace a solvent molecule in order for retention to occur by adsorption. Many of the surface hydroxyls will be inaccessible due to steric hinderance by the brush.

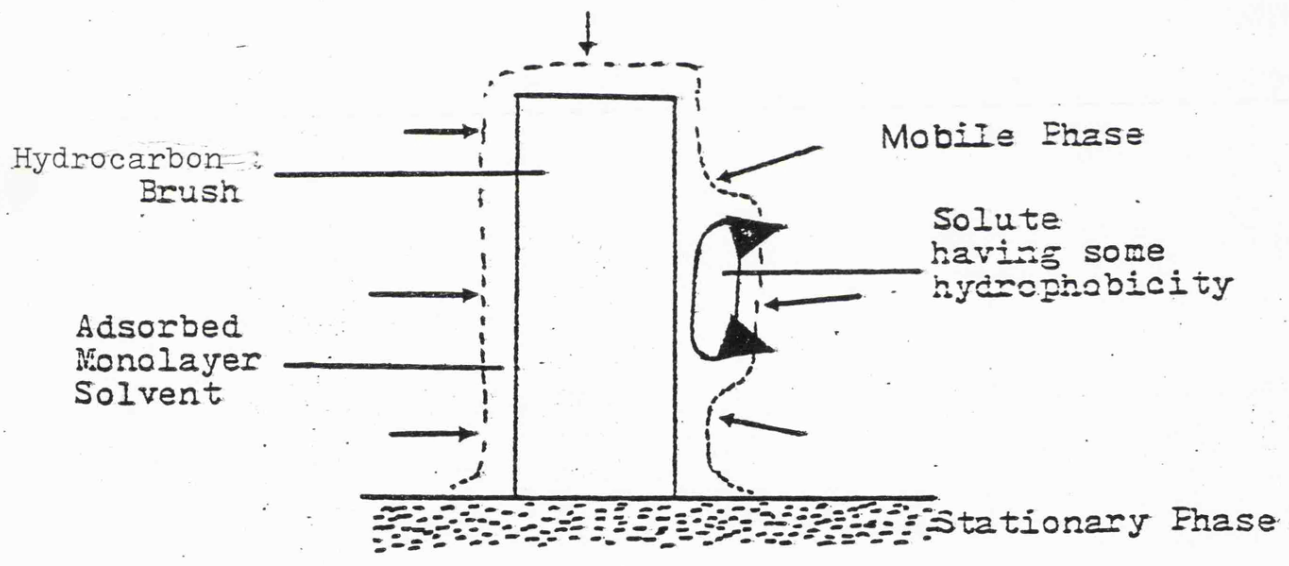
Scott and Kucera (29) have shown for methanol, acetonitrile and isopropanol that reversed phase materials adsorb a layer of solvent from the aqueous mobile phase, provided the amount of solvent in the mobile phase is enough to wet the surface of the bonded phase.

The amount of solvent adsorbed did not vary with the concentration of solvent present and calculations strongly suggested that the adsorbed solvent layer is only a monolayer.

2.3.3 Solute Interaction With the Surface of the Bonded Phase.

The same authors (29) also concluded that for solutes eluted with K' values of up to 10, the mono-molecular layer of solvent is not displaced by the solute. Thus in chromatographic development, solute retention must involve interaction with the monolayer of solvent.

FIGURE 2.8



Horvath and Melander (30A) have summarized interactions that aid retention, and these are illustrated in figure 2.8.

Interactions that aid retention of solute:-

(i) Association of brush and solute due to hydrophobic interactions causes a decrease in the molecular

surface area exposed to the solvent. Therefore an increase in % carbon loading and carbon chain length will increase retention.

(ii) Increase in hydrophobic area of solute, and

(iii) Increase in surface tension of the solvent.

Interactions that aid elution of solute:-

(i) Attractive interactions with the solvent,

(ii) A solute substituted with a polar functional group will enhance solute-solvent interactions compared to the unsubstituted form.

2.3.4 Choice of Mobile Phase.

The mobile phase in bonded phase LLC usually consists of relatively small organic molecules or a mixture of several such materials. There is a need to use low-viscosity liquids in order to minimize the driving pressure required to force it through the packed bed. An increase in mobile phase viscosity also increases the analysis time.

The solvent strengths are usually measured in terms of the solvent polarity and are presented as an eluotropic series (31-35). However, solvents in a series with comparable polarity rankings may have different solubility parameters. However, the eluotropic series is a useful means of ranking the relative polarities of solvents, and it is in common use.

Thus the most important practical point stemming from consideration of column dynamics is to choose a mobile phase of low viscosity to achieve fast analyses at modest driving pressures.

CHAPTER 3

Objectives

The mode of action of non-steroidal anti-inflammatory drugs has been linked with the inhibition of prostaglandin synthetase. This is a non-specific action and so contributes to the side effects of the drug administered. It is therefore important for patients being treated with these non-specific, high dosing drugs to be under better clinical control. Thus there is a need for a rapid technique of assessment of these agents in body fluids and suitable for routine use in clinical situations.

The wide range of non-steroidal anti-inflammatory agents available often leads to multiple drug therapy with potentially dangerous consequences. A selective method of monitoring two or more of these drugs simultaneously would be an advantage. This would also reveal those patients that employ self medication with related agents such as paracetamol or aspirin, without the knowledge or perhaps agreement of the clinician.

Furthermore, such a simple and accurate method of body fluid analysis is likely to improve our knowledge of pharmacokinetic parameters of each drug in the patient, and this could then be related to therapy. The result would be

(i) Optimization of the dose for each patient,

thus hopefully minimizing the side effects, and

(ii) An indication of the drug of choice for a specific patient.

3.1 Drugs Studied.

The non-steroidal anti-inflammatory agents chosen for this study were selected because they are being employed, or are undergoing clinical trials, at the Royal National Hospital for Rheumatic Diseases, Bath. All the clinical samples of urine and plasma were obtained from the patients in this hospital.

3.1.1 Arylcarboxylic Acids:-

(i) Alclofenac: 4-Allyloxy-3-chlorophenylacetic acid (35).

(ii) Benoxaprofen: (±)2-(4-chlorophenyl)-α-methyl-5-benzoxane acetic acid (36,37).

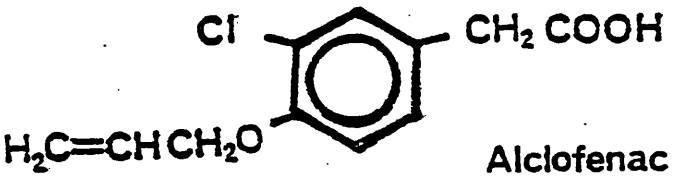
(iii) Diflunisal: 5-(2,4-difluorophenyl) salicylic acid (38,39).

(iv) Fenoprofen: (±)2-(3-phenoxyphenyl) propionic acid (40.)

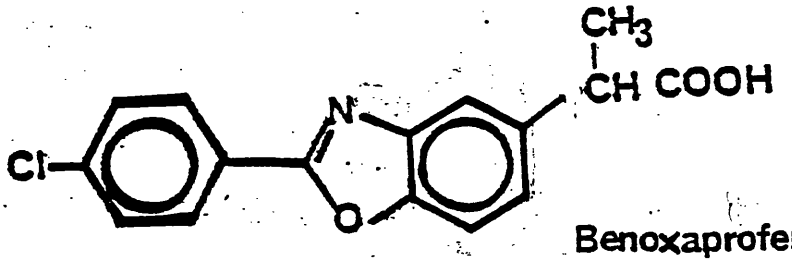
(v) Indomethacin: 1-p-chlorobenzoyl-5-methoxy-2-methyl-indoleacetic acid (15,41).

(vi) Ketoprofen: (±)2-(3-Benzoylphenyl)propionic acid (42,43).

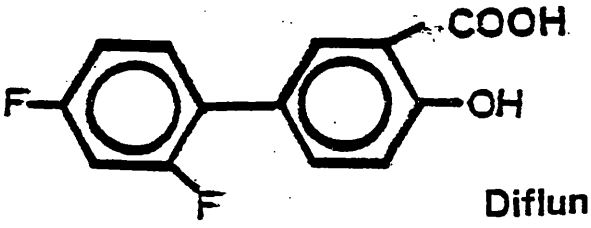
FIGURE 3.1A Structures of the Drugs.



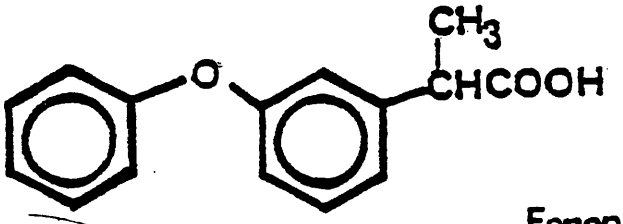
Alclofenac



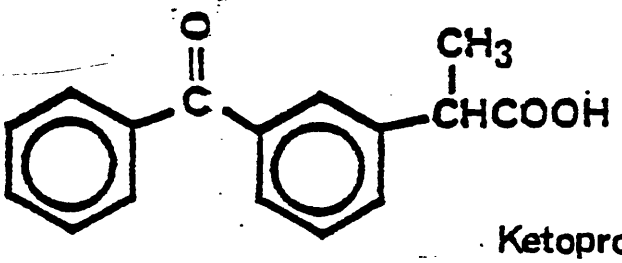
Benoxaprofen



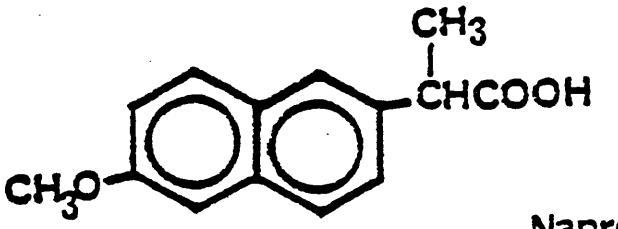
Diflunisal



Fenoprofen



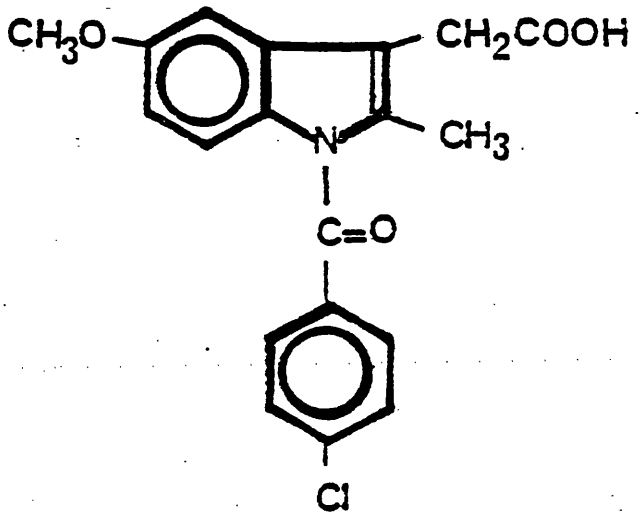
Ketoprofen



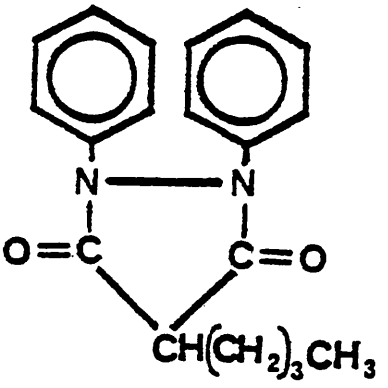
Naproxen.

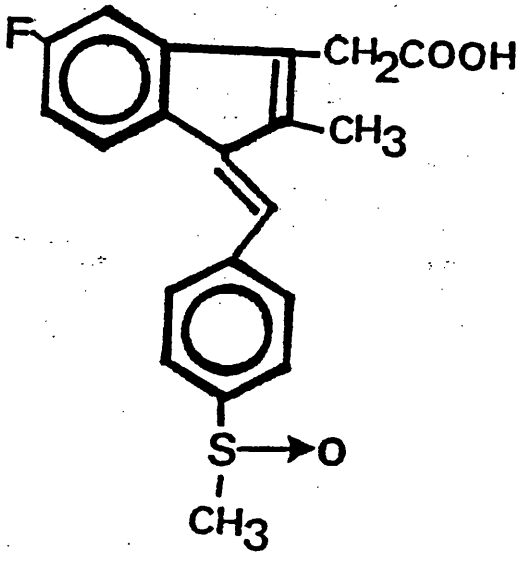
FIGURE 3.1B Structures of the Drugs (cont'd.)

Indomethacin.



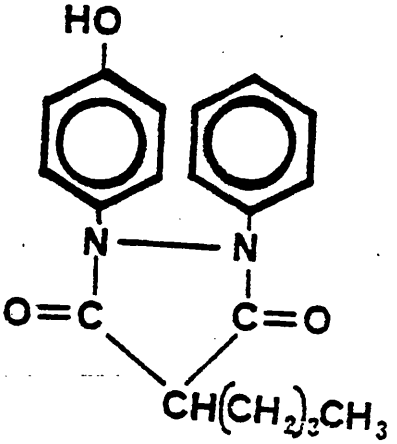
Phenylbutazone





Sulindac

(Sulphoxide)



Oxyphenbutazone

(vii) Naproxen: (+)6-Methoxy-α-methyl-2-naphthalene acetic acid (9,10,44).

(viii) Sulindac: (Z)-5-fluoro-2-methyl-1-4-methylsulphinylbenzylidene-3-acetic acid (12).

3.1.2 Amidic Acids:

(i) Phenylbutazone: 3,5-dioxo-4-n-butyl-1,2-diphenylpyrazolidine (13).

Their structures are illustrated in fig. 3.1 A&B. Physicochemically, these compounds are moderately lipophilic weak acids with pka values between 4.5 and 7.5. Their solubility in water is pH dependent, whilst they are readily soluble in organic solvents. They are also extensively bound to plasma protein. Compilation of the physical data for these compounds have already been published (45).

They all possess strongly absorbing UV chromophores, and have absorption maxima between 240 and 320mm. The absorption is enhanced in acidic conditions, because they exist in these non-ionic forms, and at 254mm, it is considerable for all of them. A variable wavelength UV-monitor is therefore the detector of choice.

3.2. Choice of Technique.

HPLC appeared to be the technique of choice because it has inherent advantages over gas chromatography, which are;

(1) A wide range of stationary and mobile phases which improves the possibility of developing a selective and rapid procedure.

(2) The possibility exists for minimizing sample preparation time because:-

(i) Derivatization may not be required, and

(ii) Direct injection of urine and de-proteinated plasma may be possible.

(3) Operating at ambient or slightly higher temperatures means that thermally sensitive solutes are less likely to be decomposed during the analysis. In GC., the possibility exists for some decarboxylation of these non-steroidal anti-inflammatory agents to occur because of the high temperatures employed.

The developed procedure must be sufficiently sensitive in order to be able to accurately quantify plasma and urine levels of the drugs and their metabolites. This should be applicable both to the high doses encountered in chronic situations, and also to single dose studies encountered in bio-availability studies.

The variable wavelength ultra-violet monitor would seem to be suitable for the detection of nanogram quantities of these compounds, and so meet these requirements.

The objective of this work was therefore to develop selective and rapid analytical HPLC procedures suitable for routine use in clinical situations. In this way, a contribution could be made towards the study of drug metabolism and availability in individual patients, with a view to improving the drug therapy they receive.

RESULTS AND DISCUSSION

CHAPTER 4

The Development of HPLC Procedures

Silicagel has proved to be a highly successful material for the separation of non-ionic compounds both by thin-layer chromatography and by column chromatography. But the mechanism of adsorption is less suitable for water-soluble and ionisable compounds. Liquid-liquid column chromatography employed partition as the retentive mechanism, but suffered from the serious disadvantage of requiring the mobile phase to be saturated with the stationary phase. The development of chemically bonded phases has completely overcome this problem. The reversed phase material in particular has proved to be a remarkably versatile material, and has been the subject of many papers (46,47). The reason for this versatility was not known at the start of these studies, although it was known that retention increased with increasing hydrocarbon chain length (48). With regard to selectivity, the approach adopted by most workers has been the modification of the mobile phase to vary retention. Locke (49) proposed that the eluent order of solutes is the order of solute solubility in the mobile phase, i.e. decreasing polarity.

The retention mechanism of reversed phase chromatography then was that the hydrophobic surface attracts the more lipophilic component of the eluent to form an organic

solvent rich layer at the hydrocarbon surface. The retention of various members within a class of compounds has been correlated to their solubility in the eluent. The early studies reported on the applications of reversed phase chromatography were:

(1) Ion pair partitioning chromatography of:-

(a) Biogenic amines and their metabolites (50),

(b) Tricyclic psychosedative drugs (51).

(2) Soap chromatography for the separation of ionizable materials (28).

Twitchett and Moffat in 1975 (52) evaluated the performance of a reversed phase, (octadecylsilane, ODS), stationary phase for 30 compounds and found their chromatographic behaviour to be predictable on the basis of pKa and partition coefficient. It was also found that the stationary phase was valuable for the separation of acidic and neutral drugs, but poor for basic drugs.

The versatility of the reversed phase material as demonstrated by the above workers led to its choice as a possible material for the analysis of anti-inflammatory agents based on their physicochemical properties, eg., pKa.

The profile study of these drugs shows that they are compounds of molecular weights between 200 and 400. They are structurally similar and classified as carboxylic and amidic or enolic acids. They are moderately lipophilic

and weakly acidic, with pKa's between 4.5 and 7.5 and are extensively bound to plasma protein. These agents are partially soluble in water, readily in organic solvents and their solubility in aqueous medium is governed by pH (45). At pH 7.5, most of these compounds are expected to be significantly ionized, while at pH 3, negligible ionization is expected (figure 4.1).

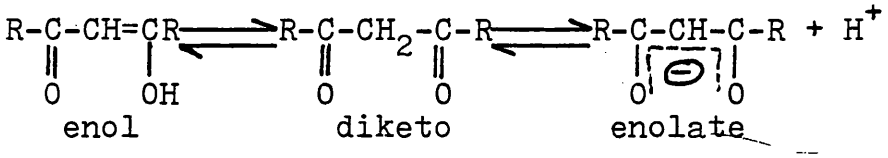
FIGURE 4.1

Ionization Process for the Acidic Protons In:-

(1) Carboxylic Acids:-

$$R\ COOH \underset{pH \leq pKa-2}{\overset{pH \geq pKa-2}{\rightleftharpoons}} R\ COO^- + H^+$$

(2) Amidic Acids:-



The choice of an aqueous organic solvent might selectively solvate the solutes and result in their selective retention.

4.1 The Initial Chromatogram.

Five of the anti-inflammatory agents were chosen to form a test mixture so that the selectivity of the chromatographic conditions investigated might be assessed. This was considered to be important because it was hoped that the same basic method would be suitable for all of them. It would also probably permit the analysis of any one of them in the presence of another anti-inflammatory agent.

Various aqueous acetonitrile mobile phases did not give a satisfactory separation. However, good resolution was achieved with aqueous methanol mixtures, especially between compositions of 30 to 50% methanol. It was found that symmetrical peak shapes could be obtained for all the agents only when the pH of the solvent was adjusted to 3, figures 4.2A&B. Analysis times were shortened and peak shapes improved by changing the column length from 200 to 50mm., with equally good resolution.

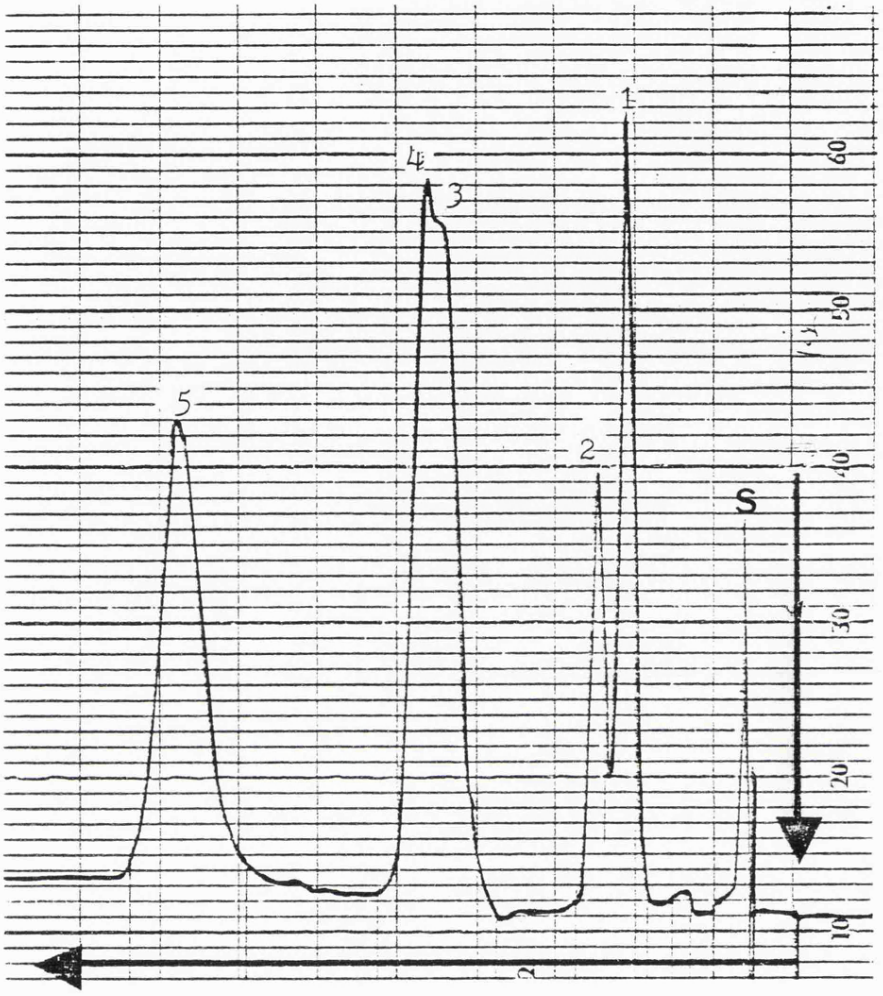
The retention could therefore be explained in terms of solubility in the mobile phase with elution in order of their decreasing polarity, i.e. most polar eluted first. The solubility is influenced by:-

(i) The % of methanol in the mobile phase, and

(ii) The pH of the mobile phase.

The system is pH dependent because of its effect upon ionization of the solutes. In reversed phase chromatography, when a dilute solution of ionizable solute is passed

FIGURE 4.2A Separation of a Test Mixture



HPLC Conditions.

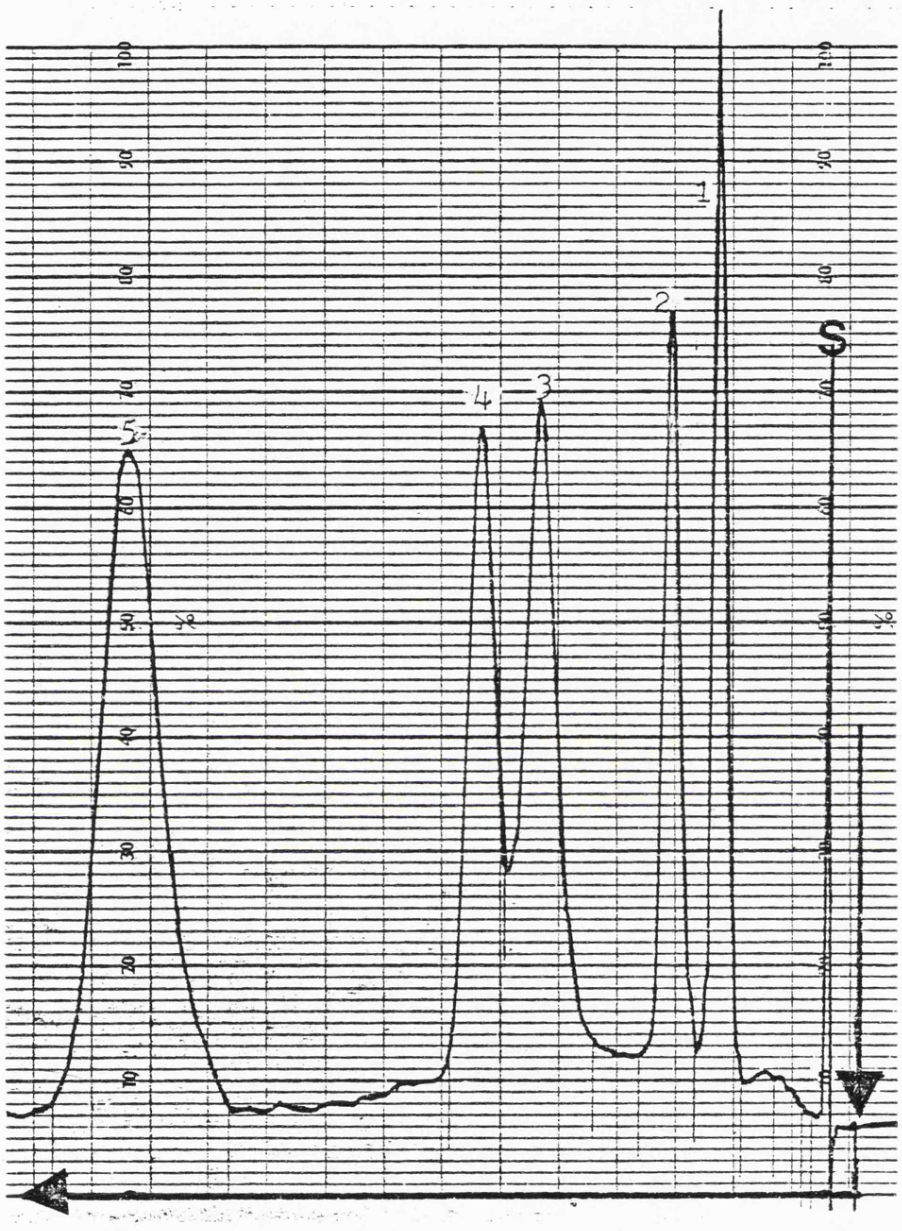
Column:- Spherisorb 5-ODS (50x4.6mm id)

Mobile Phase - Methanol:Water (45:55) pH.3

Detector λ - 254nm

Test Mixture - S-Solvent
1- Alclofenac
2-Ketoprofen
3-Fenoprofen
4-Phenylbutazone
5-Indomethacin

FIGURE 4.2B Separation of the Same Mixture As in 4.2A



HPLC Conditions.

Column:- Spherisorb 5-ODS (50x4.6mm Id)

Mobile Phase - Methanol:Water pH 3. (35:65)

Detector λ - 254nm

Test Mixture - S-Solvent
1-Alclofenac
2-Ketoprofen
3-Fenoprofen
4-Phenylbutazone
5-Indomethacin

through a hydrophobic organic stationary phase, the solute will be distributed between the two phases, figure 4.3.

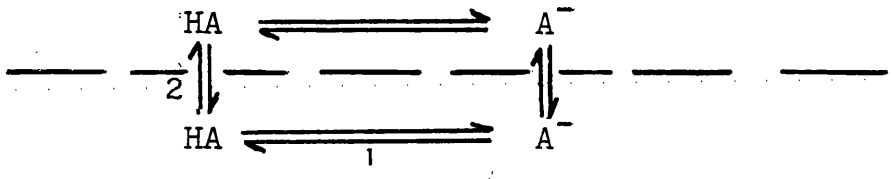
A decrease in ionisation produces a decrease in the hydrophilic interactions with the aqueous mobile phase, thereby increasing retention on the hydrophobic stationary phase.

When pH = pKa-2, the ionisation of the solute is completely suppressed and the solute has a maximum capacity ratio. Under this condition of 'ionic suppression', the system is very selective for the solute, based upon its distribution between the hydrophobic stationary and hydrophilic mobile phase.

The appreciation of these factors led to an investigation into their influence upon the retention of each of the drugs studied.

FIGURE 4.3 Distribution of Solute HA.

Stationary phase: Hydrocarbon + adsorbed organic modifier (MeOH)



Mobile phase: Aqueous Organic Solvent (H_2O + MeOH)

Equilibrium 1 is pH dependent and the degree of ionization is given by the Handerson-Hasselbalch equation, where

$$pH = pKa + \log\left[\frac{A^-}{HA}\right]$$

Equilibrium 2 represents the distribution of the non-ionised form HA.

The ratio of the concentration of the non-ionised form in each phase is a function of:

1. The pKa of the solute,
2. The pH of the aqueous phase, and
3. The distribution coefficient of the neutral, non-ionised form between the stationary and mobile phases.

4.2 The Effect of Organic Modifier Upon Retention and Selectivity.

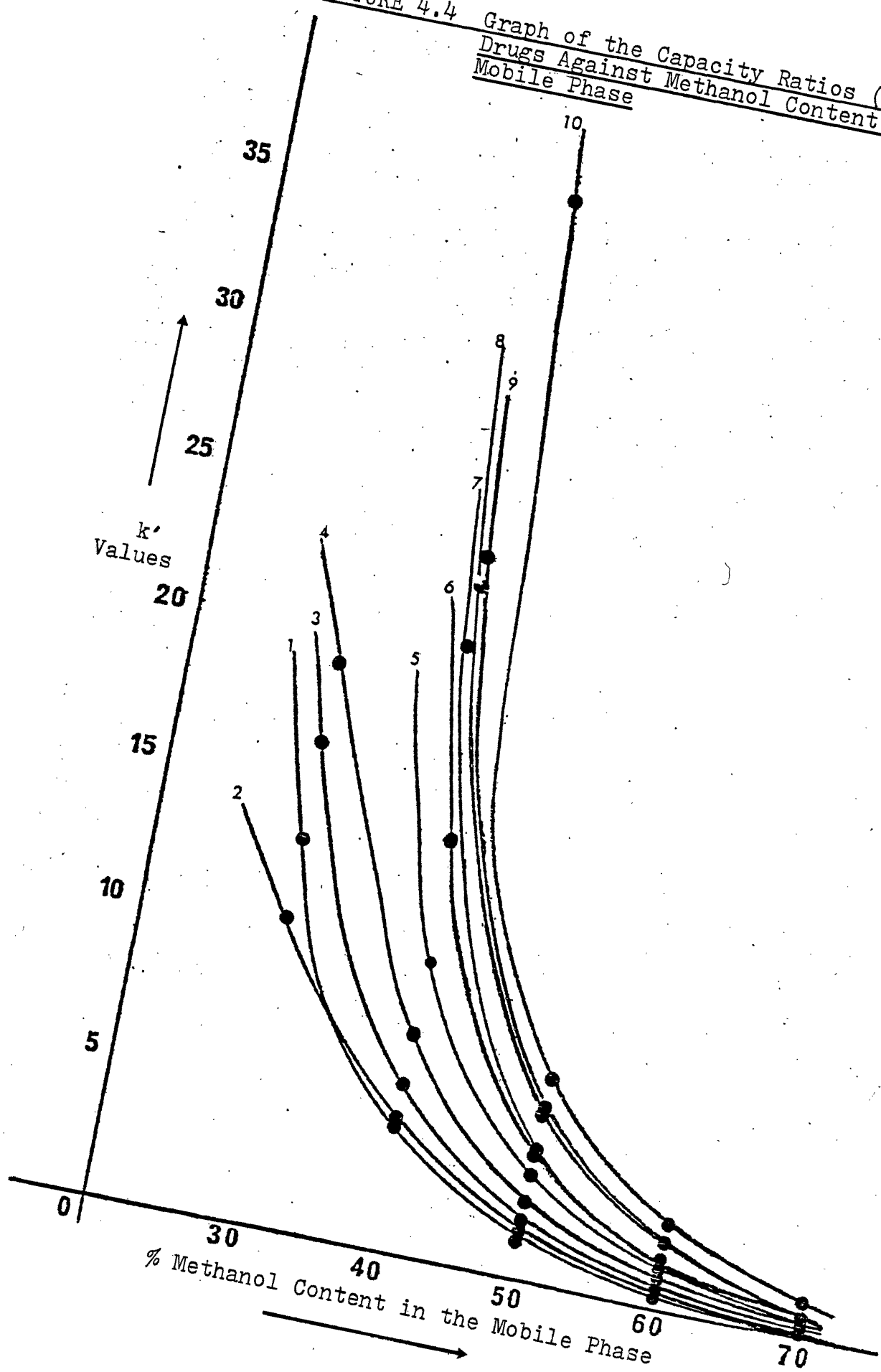
The effects upon retention for the ten anti-inflammatory agents are given in Table 4.1 and illustrated in Figure 4.4. It can be seen that, as expected, an increase in methanol concentration decreases the K' values for all the solutes. A change in elution order was observed only between the two compounds having the lowest K' values, namely, oxyphenbutazone and alclofenac. In general, no change in elution order occurred.

The selectivity of the conditions was measured by comparison of K' values for adjacent peaks at the various methanol concentrations. Selectivity is more informative when measured using a parent compound compared to a range of substituted derivatives. In this way, selectivity towards individual substituent groups is obtained. However, the series of compounds available here did not permit this treatment because of their differences in chemical structures. The data in Table 4.1 does show that for most adjacent peaks, selectivity is reduced as retention falls, as illustrated by the pairs (2,3), (4,5) and (6,7). It is also clear that selectivity towards all the compounds is good at 50% methanol, and can be rapidly improved by small reductions in methanol concentration.

TABLE 4.1 Capacity Ratios (k′) and Selectivity (α) of Anti-Inflammatory Agents Measured With Different Methanol-Water Mixtures As Mobile Phase.

	Drugs	Mobile Phase									
		30:70		40:60		50:50		60:40		70:30	
		k′	α	k′	α	k′	α	k′	α	k′	α
1	Oxyphenbutazone	13.00		4.33		1.33		0.67		0.00	
			0.80		1.08		1.25		0.77		
2	Alclofenac	10.33		4.67		1.67		0.87		0.33	
			1.58		1.21		1.20		1.00		1.00
3	Ketoprofen	16.33		5.67		2.00		0.87		0.33	
			1.14		1.29		1.17		1.15		1.67
4	Naproxen	18.67		7.33		2.33		1.00		0.50	
			-		1.59		1.51		1.33		1.00
5	Fenoprofen	-		11.67		3.53		1.33		0.50	
			-		1.20		1.13		1.00		1.20
6	Phenylbutazone	-		14.00		4.00		1.33		0.60	
			-		1.50		1.00		1.00		0.83
7	Sulindac	-		21.00		4.00		1.33		0.50	
			-		1.08		1.42		1.50		1.33
8	Indomethacin	-		22.67		5.67		2.00		0.67	
			-		1.03		1.06		1.00		1.00
9	Diflunisal	-		23.33		6.00		2.00		0.67	
			-		1.53		1.50		1.34		2.00
10	Benoxaprofen	-		35.67		9.00		2.67		1.33	

FIGURE 4.4 Graph of the Capacity Ratios (k) of the Drugs Against Methanol Content in the Mobile Phase



4.3 Effect of pH on Retention

At a constant eluent strength of methanol-water (50:50), the effects of varying the pH of the eluent was observed on the retention of the agents (Table 4.2).

TABLE 4.2 Effect of pH on Capacity Ratios (K')

	Drugs	K' Values at pH		
		2.5	6.0	7.5
1.	Oxyphenbutazone	1.33	0.50	0.10
2.	Alclofenac	1.67	0.75	0.15
3.	Ketoprofen	2.00	1.25	0.21
4.	Naproxen	2.33	1.38	0.28
5.	Fenoprofen	3.53	1.25	0.25
6.	Phenylbutazone	4.00	2.13	0.43
7.	Indomethacin	4.00	3.00	1.14
8.	Sulindac	5.67	3.00	0.60
9.	Diflunisal	6.00	2.00	0.40
10.	Benoxaprofen	9.00	7.36	1.68

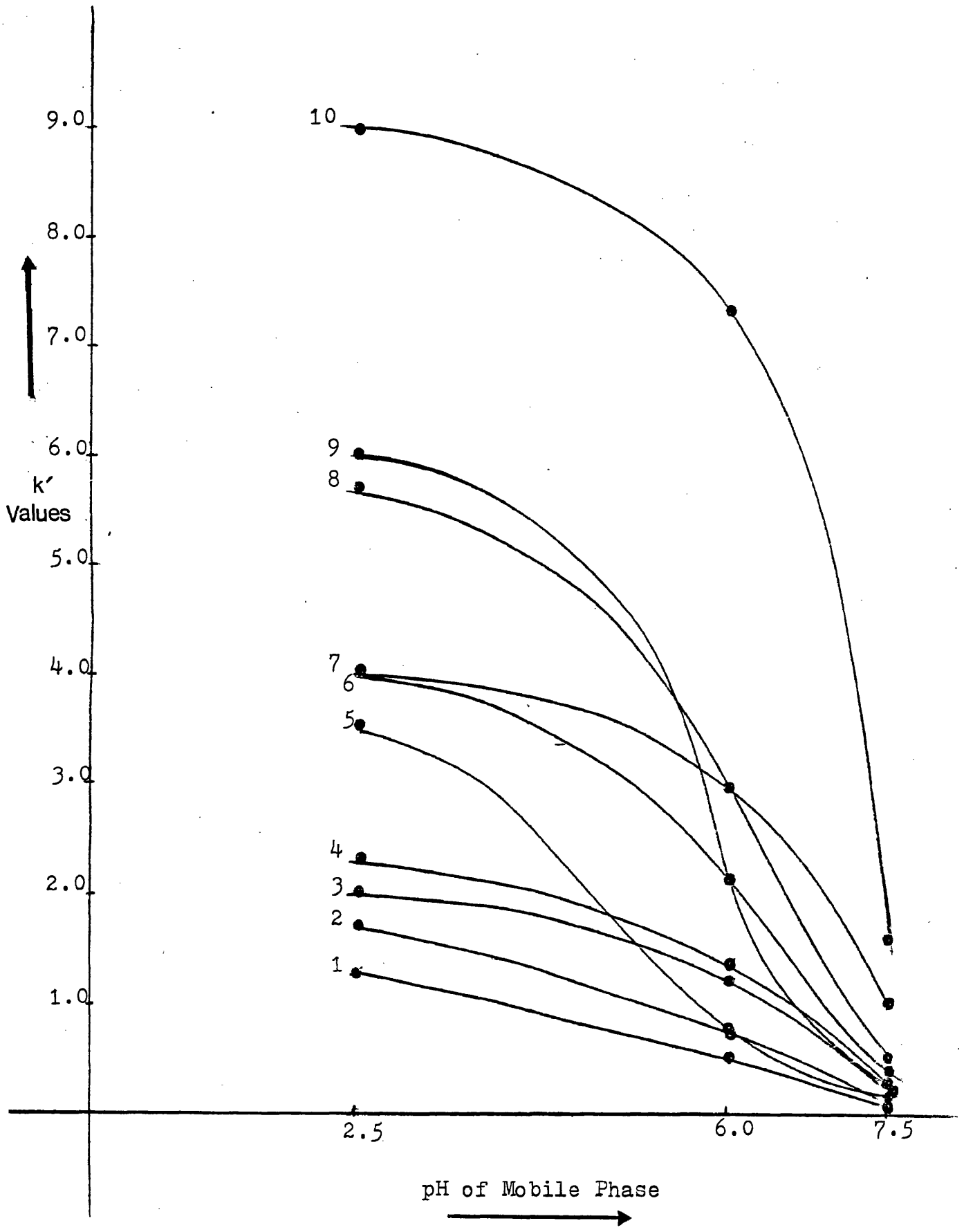
Column: Spherisorb 5-ODS (50x4.6mm id)

Mobile Phase: Methanol:Phosphate Buffer (50:50)

Detection: 254nm

The results are further illustrated in figure 4.5.

FIGURE 4.5 Graph of the Capacity Ratios (k') of Anti-Inflammatory Agents.



Usually, the ionic solutes are not absorbed at all by the non-polar stationary phase, because highly polar or ionic solutes interact strongly with the aqueous solvent and are easily solvated (30B).

From the graph, there is a marked decrease in the retention of the drugs when the pH rises above 3 where ionization of the solutes begins and the order of retention is changed. When this occurs, K' values are generally less than 3, which is too small for analytical studies in the presence of biological fluids. It is apparent therefore that no improvement in selectivity can be obtained by choosing a pH at which ionization of the compounds occurs, and that retention is more conveniently controlled by changing the methanol concentration. Ionization of the solutes causes the solutes to interact strongly with the aqueous mobile phase and are thus easily solvated. This makes the solutes less retained.

4.4 Ion-Pair Chromatography.

An alternative approach to the enhancement of retention of ionizable compounds is to use ion-pair chromatography. This technique involves the addition of an oppositely charged pairing-ion to the chromatographic system, so that solute retention is effected by ion-pairing by various mechanisms. Schill, Persson and co-workers (53-55) pioneered this technique for normal phase chromatography, in which the pairing ion is loaded on to a hydrophilic stationary phase such as silica gel. In a recent application of this technique, Schill (56) used perchlorate as the pairing ion and separated two amines, zimelidine and demethylzimelidine from plasma using methylene chloride and n-butanol as mobile phase. However, solutes must be dissolved in the mobile phase before injection, and meticulous thermostatting of the chromatographic system is necessary for column stability. The method has been used by other workers such as Persson and Karger (50) for biogenic amines, and Knox and Jurand (51) for tricyclic tranquilizers. It is also an excellent method for solutes having little or no UV absorbance.

In 1975, Wittmer, et al. (57) used a reversed phase column with a mobile phase of aqueous methanol containing tetrabutylammonium hydroxide as pairing ion. This enabled a very polar sulphonic acid dye tartrazine, and its

intermediates, to be adequately retained for good resolution. In 1976, Knox and Laird (28) introduced "Soap Chromatography" to describe their studies using cetyltrimethylammonium bromide (cetrimide) as a pairing ion in both reversed phase and silica gel columns. The solutes examined were also a range of sulphonic acid dyestuffs, which had given tailing peak shapes using conventional adsorption or normal phase ion-pair chromatography using tetraalkylammonium pairing ions. This was attributed to the very strong polarity of the $-SO_3-$ group displacing water from the silanol sites on the silica surface, even in the presence of a strongly aqueous mobile or stationary phase. However, they found that highly efficient columns were obtained ($h \triangleq 2x$ particle diameter) in the presence of cetrimide for both reversed phase and silica gel columns, which is comparable to that obtainable in normal adsorption chromatography. In the reversed phase mode, the retention mechanism was considered to be probably a combination of two mechanisms:-

(1) Formation of the cetrimide-sulphonate ion-pair in the mobile phase, followed by distribution into the hydrophobic stationary phase.

(2) Ion-pair formation between cetrimide adsorbed into the hydrophobic stationary phase and sulphonate ions in the mobile phase.

From the many papers published since 1976 on this topic and recently reviewed (58), it would seem probable that both mechanisms frequently occur simultaneously, and that the percentage of organic modifier is a key factor in determining which retention mechanism will dominate (29). In spite of the detailed studies of Horvath and co-workers (30B), the various equations describing the equilibria involved when used with experimental data are unable to distinguish between the two mechanisms.

In view of the excellent column efficiencies obtained with cetrimide, it was decided to investigate the suitability of this pairing-ion for non-steroidal anti-inflammatory agents. The results are tabulated in Table 4.4 and illustrated in figure 4.6. Although the pH of 7.5 was not sufficiently high to ensure complete ionisation of all the drugs studied, it was used because it was the highest value recommended for silica-gel.

It was found that K' values increased rapidly in the presence of cetrimide, and were excessively so (>10) for the most hydrophobic agents, namely sulindac, indomethacin, diflunisal and benoxaprofen. For the other agents, at a pairing ion concentration of 2×10^{-4}M, K' values were generally more than doubled with little change in selectivity, when compared with ionic suppression as shown in Table 4.1 .

TABLE 4.4 Effect of Concentration of Cetrimide in the Mobile Phase on Retention of Anti-Inflammatory Agents.

	DRUGS	Mobile Phase : Methanol : Phosphate Buffer 50:50 pH 7.5									
		Cetrimide Concentrations									
		No Cetrimide		10^{-4}M		$2x10^{-4}$M		$4x10^{-4}$M		$6x10^{-4}$M	
		K'	α	K'	α	K'	α	K'	α	K'	α
1	Oxyphenbutazone	0.10		0.50		2.50		3.75		4.25	
			1.50		1.50		1.95		2.33		2.35
2	Alclofenac	0.15		0.75		4.88		8.75		10.00	
			1.40		1.67		1.23		1.11		1.08
3	Ketoprofen	0.21		1.25		6.00		9.75		10.75	
			1.33		1.10		1.23		1,18		1.14
4	Naproxen	0.28		1.38		7.38		11.50		12.25	
			1.12		1.62		1.05		1.11		1.16
5	Fenoprofen	0.25		2.03		7.75		12.75		14.25	
			1.72		1.05		1.50		1.37		1.30
6	Phenylbutazone	0.43		2.13		11.60		17.50		18.50	
			1.40		2.58		1.13		0.05		1.25
7	Sulindac	0.60		5.50		13.13		20.75		23.25	
			1.90		1.09		2.51		-		-
8	Indomethacin	1.14		6.00		33.00		-		-	
			2.85		1.21		2.28		-		-
9	Diflunisal	0.40		4.98		14.50		31.50		52.75	
			4.20		1.68		2.62		-		-
10	Benoxaprofen	1.68		8.38		38.00		-		-	

FIGURE 4.6 Graph of Capacity Ratios (k') Against the Concentration of Cetrimide in the Mobile Phase

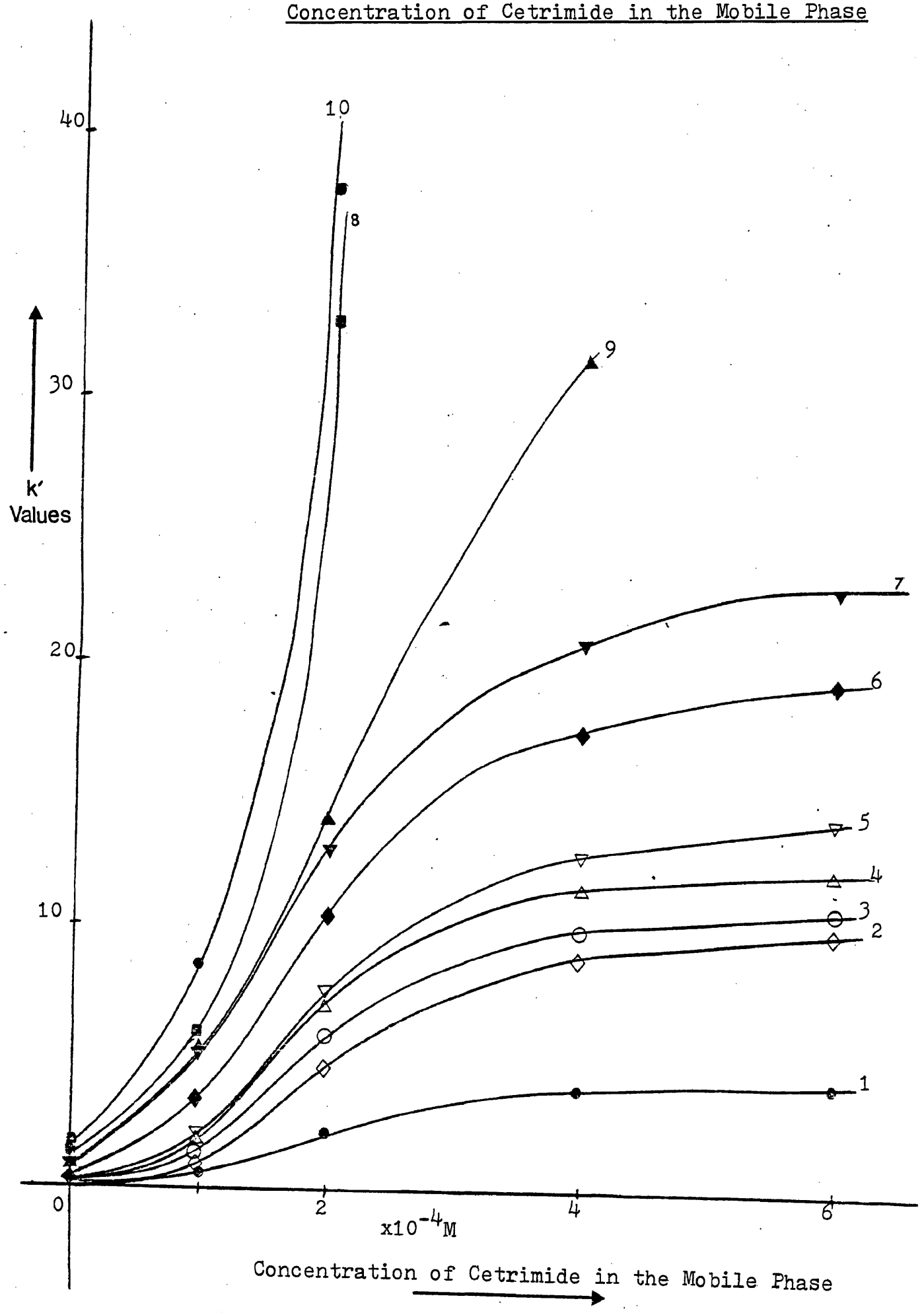


Figure 4.6 illustrates that the relationship between retention and pairing-ion concentration is typically sigmoidal, as has been shown by other workers (59). This is thought to be due to the depletion of the pairing-ion by the solute at very low concentrations. Thus the optimum pairing-ion concentration to use would be 1.5 to 4 x 10^{-4}M, i.e. the rising portion of the curve. The elution order of the compounds was not altered by variations in pairing-ion concentration, which is in agreement with the work of Johansson, et al. (60).

The formation of the ion-pair may be described by the equation:

$$C_{16}H_3N^{+}(CH_3)_3Br^{-} + RCOO^{-}H^{+}$$

cetrimide ↓ anti-inflammatory acid

$$\left[C_{16}H_3N^{\delta+}(CH_3)_3,\ RCOO^{\delta-}\right]$$

ion pair

The equilibria involved in the mechanisms of retention by reversed phase ion-pair chromatography have been described by Horvath and co-workers (30B) as

$$\begin{array}{ccccc} E + H & \overset{K_2}{\rightleftharpoons} & EH & \overset{K_2}{\rightleftharpoons} & E^{+} H \quad \text{mobile phase} \\ \Big\Updownarrow K_1 & & \Big\Updownarrow K_4 & & \Big\Updownarrow K_3 \\ LE + H & \overset{K_6}{\rightleftharpoons} & LEH & \overset{K_5}{\rightleftharpoons} & E + LH \quad \text{stationary phase} \end{array}$$

where E = solute = anti-inflammatory acid

H = pairing-ion

L = hydrocarbon surface.

The two retention mechanisms may be represented by the equilibria:-

(a) K_2K_4 = Ion-pair formation in the mobile phase followed by transfer to the stationary phase, and

(b) K_3K_5 = Ion-pair formation between adsorbed pairing ion and anti-inflammatory agent in the mobile phase, also known as in-situ ion exchange.

When control plasma and urine samples were injected into the cetrimide system, it was found that the elution volume required for the peaks to elute was much increased because these materials normally contain endogenous acidic compounds capable of forming ion-pairs with the cetrimide, so increasing their retention, when compared with those obtained using ionic suppression.

Another feature of the ion-pair system was that at pH 7.5, the anti-inflammatory agents exhibited a lower absorbance in UV light so that sensitivity of detection was impaired.

In view of results obtained with cetrimide, it was decided to adopt the ionic suppression system as the method of choice.

4.5 Conclusion.

4.5.1 Significant Features of the HPLC Technique.

The HPLC procedures developed and used in these studies have been based upon reversed phase chromatography utilizing ionic suppression of the solute. The column capacity ratio of a solute is a measure of the balance between retention caused by hydrophobic interactions of the solute-stationary phase complex and elution caused by interactions between solute functional groups and organic modifier. This is capable of producing a wide range of K' values responsible for the significant features of this method, namely:

1. Flexibility,
2. Load capacity,
3. Selectivity.

4.5.2 Flexibility.

The inherent advantages and applicability of this method can be examined by comparison with gas chromatography and thin-layer chromatography. Thin-layer chromatography offers several advantages over HPLC in that:-

(i) The technique is simple and inexpensive,

(ii) A number of samples can be separated simultaneously on the same layer,

(iii) The separation can be carried out in two dimensions with two different eluents, and

(iv) No components are overlooked, since the sample is detected on the chromatographic system.

However, the disadvantages are:-

(i) The precision of quantitative analysis is moderate,

(ii) The precision of the migration data for identification is moderate,

(iii) The method is not favourable for automation, and

(iv) The number of theoretical plates is limited by the fact that the fluid velocity decreases to zero with time.

Gas chromatography also has some advantages over liquid chromatography in that:-

(i) In general, for solutes having a K' value greater than about 10 or 20, G.C. produces better peak shapes than HPLC.

(ii) Extremely high plate numbers may be obtained using capillary columns.

(iii) Detectors in GC are much more sensitive. The flame ionization detector, FID, available in GC is very sensitive, with a detection limit of about 10^{-9}gm, i.e. in the ng range, and responds to almost all organic compounds.

The electron capture detector is about 100 times more sensitive to halogenated compounds or derivatives. For HPLC the UV variable wavelength detector is suitable for organic compounds having conjugated double bonds, and is sensitive in the ng range. Fluorimetry is also available with at least a x10 improvement in sensitivity.

But on the other hand, HPLC offers several distinct advantages over GC, including:-

(i) The ability to analyse material of very low vapour pressure without the preparation of volatile derivatives,

(ii) Analyses of thermally unstable materials may be accomplished with ease, since the technique can be operated at room temperature.

(iii) The polarity of the mobile phase compared with stationary phase is readily variable to provide a wide range of different conditions capable of achieving a separation.

The method of reversed phase chromatography using hydrocarbonaceous stationary phases with hydro-organic solvent mixtures has proved to be extremely versatile, and probably accounts for 60-80% of all applications published recently. The features of reversed phase HPLC responsible for its flexibility include:-

I. An aqueous mobile phase which provides:

1. Compatibility with biological fluids, since most biomolecules are compatible with such an environment, solubility can be readily achieved.

2. Operational simplicity by the use of aqueous organic solvents, thus avoiding dangerous and toxic solvents.

3. Selective equilibria such as ionization (61,62), ion pair (55,58), and ligand exchange (63) may be employed, creating special opportunities for ionizable substances.

However, the presence of water is a major disadvantage when HPLC is linked to Mass Spectrometry. Analyses have been achieved on HPLC-MS using a mobile phase containing not more than 40% water (64).

4. Good column stability,

(a) Water or biological fluids in the injection solvent will not influence retention because water is already present in the mobile phase. In contrast, traces of water in the injection solvent will cause retention variations with silica gel columns, as used in adsorption chromatography.

(b) Highly polar impurities or metabolites frequently elute unretained, whereas they accumulate in adsorption chromatography and are retained on the column, gradually degrading its performance.

II. Weak surface energies of bonded alkyl phase.

This permits not only rapid analyses but rapid equilibrium when eluents are altered. About 10-25 column volumes were necessary for equilibrium to be reached on reversed phases, whereas about 50-100 or more are required for silica. For the same reason, not only aqueous solutions, but protein or particle free biological fluids can be injected directly into reversed phase columns.

III. Application to Physico-chemical Studies.

Reversed phase systems have also been used as a physico-chemical tool for characterization of biochemical substances, eg., comparing fundamental properties of molecules and their metabolites for their hydrophobicity (65) (Chapter 5).

The current limitations in reversed phase HPLC are:-

(1) Stable columns can be obtained only between the pH range of 2 to 7.5. At low pH, attack of the Si-C-hydrocarbon bond is possible whereas at high pH, the siloxane structure of silica may be attacked, particularly in salt solutions. Most of the analyses in this work were achieved at pH of between 2.5 and 3, and columns were found to be stable and with good performance for over six months. However, regular regeneration of columns with methanol was necessary.

(2) The remaining silanol groups (Si-OH) on the silica surface, figure 2.7, often influence the retention of polar, and particularly basic compounds. If these silanol groups are accessible for interaction, then retention will be affected and peak tailing may result.

(3) The variety and quality of commercially available reversed phase materials have lacked uniform performance, although the order of elution is usually identical under the same conditions, figure 7.6.

4.5.3 Load Capacity.

Unlike gas chromatography, HPLC can be used for semi-preparative separations without instrument modification. It means that a peak of interest may be collected, from a number of injections and then subjected to further study. For example, ketoprofen glucuronide (Chapter 5) was collected, hydrolysed, and the presence of ketoprofen confirmed by HPLC. For preparative studies, columns of wider internal diameter (8mm.id.) and longer lengths (200-300mm) are often used.

4.5.4 Selectivity.

This is the intrinsic capability to distinguish between two components. The separating power of reversed phase system arises from:

(i) The control of mobile phase, and

(ii) The performance of the column.

(i) Control of Mobile Phase:-

The effect of solvent strength for all the samples was always investigated as demonstrated earlier in this chapter (2.1.2.2) before the optimum eluent strength was logically chosen. This is because solvent strength is the sum total of three types of intermolecular interactions acting concurrently, eg., dispersion, orientation and hydrogen bonding (66). Each solvent has these interactive components in a unique ratio. Thus, two solvents of approximately equal polarity can have different interactive profiles. Solutes also have a profile, and when there is a good interactive match between solvent and solute, the solvent strength is particularly high. Selectivity is thus the degree to which a solvent is chromatographically stronger for a particular solute by virtue of its ability to enter into specific intermolecular interactions to a greater degree than for other solutes.

Reversed phase systems in these studies have used water as a mobile phase component. It is a unique solvent with a high cohesive energy density which produces a high surface tension. All but the most polar or ionic solutes experience a hydrophobic force that drives them onto the stationary phase and causes retention (67). The hydrophobic effect results from the strong attractive forces

between water molecules. Highly polar or ionic solutes can themselves interact strongly with water and are thus easily solvated, but non-polar solutes are relatively insoluble.

The solute is retained in the hydrophobic stationary phase coated by a monolayer of the organic modifier, figure 2.8. The hydrophobic force of water isusually reduced by adding miscible organic solvent. Methanol has proved to be the solvent of choice in these studies largely because its polarity renders it a good solvent for acidic anti-inflammatory agents. It is stable with a low viscosity, and does not present a serious health risk to the user.

Thus, the technique of HPLC is ideal for the analysis of acidic anti-inflammatory agents either alone or in combination and possesses many advantages over previously reported methods.

CHAPTER 5

Applications of HPLC Procedures Developed.

The preliminary studies had provided two chromatographic systems, namely ionic suppression and reversed phase ion-pair chromatography that could be used in the analysis of these anti-inflammatory agents. The choice between the two has been based on:-

(i) The ability to control the ionization of the solutes at the selected pH;

(ii) The stability of the drugs at the working pH over a fairly long period of time;

(iii) The sensitivity or the coefficient of extinction of the agents in UV light at the chosen pH - to aid detection;

(iv) The ease of sample preparation which controls the use of solvents and also the cost; and

(v) The analysis time, since efficient assay procedures aimed for routine work should have a short analysis time.

The advantages of using ionic suppression for the chromatographic conditions were considered to be:-

(i) Diluted control urine and deproteinated plasma was largely unretained and did not produce any peaks likely to interfere with the solutes of interest. This

enabled K' values between 4 and 10 to be chosen for the solutes, thus ensuring that analytical times would be short.

(ii) At a pH of 3, the ionization of all the drugs was adequately suppressed to provide a variation of K' values based upon their relative hydrophobicities.

(iii) Symmetrical, narrow peaks were obtainable, suitable for quantification by peak height measurement.

(iv) The drugs and column material were known to be stable at pH 3. Clinical samples are also preserved in acid medium prior to their analysis.

(v) The drugs have higher molar extinction coefficients in acid conditions than in neutral or weakly alkaline solvents, thus increasing their sensitivity to detection by UV monitors.

Karger in 1978 (68) from his previous studies and experience has also found it sometimes preferable to chromatograph a solute in its non-ionized form since there is then less chance of secondary equilibria influencing retention or band shape.

Ionic suppression mechanism on a reversed phase column using aqueous methanol at pH 3 has thus been chosen for the study of these drugs in biological fluids. In this system, more polar metabolites and impurities elute before the drug. By choosing a capacity factor K' of between 4 and 10 for the drug should clearly separate the biological fluid components and drug metabolites from the free drug.

5.1 Determination of 10 Non-Steroidal Anti-Inflammatory Agents in Plasma and Urine.

5.1.1 Sample Preparation:-

The schematic representation of the procedure is shown in figure 5.1A and B which involves extraction of the free drug from acidified aqueous plasma or urine into the organic solvent, diethylether.

5.1.2 Assay Procedure:-

2.1 HPLC Conditions.

The summary of the HPLC conditions which include the mobile phase composition, the detector wavelength, the internal standard used and the limit detected for each drug is shown in Table 5.1.

2.2 Calibration of Drugs in the Mobile Phase.

Drug concentrations in the range of 1.0 to 80µg/ml in the mobile phase were prepared by suitable dilutions of the stock solution of the drug (1mg/ml) in the mobile phase. Calibration plot of the peak height ratio of the drug to the internal standard vs the concentration of the drug per ml of the mobile phase was then constructed.

FIGURE 5.1A

EXTRACTION OF DRUGS FROM SERUM.

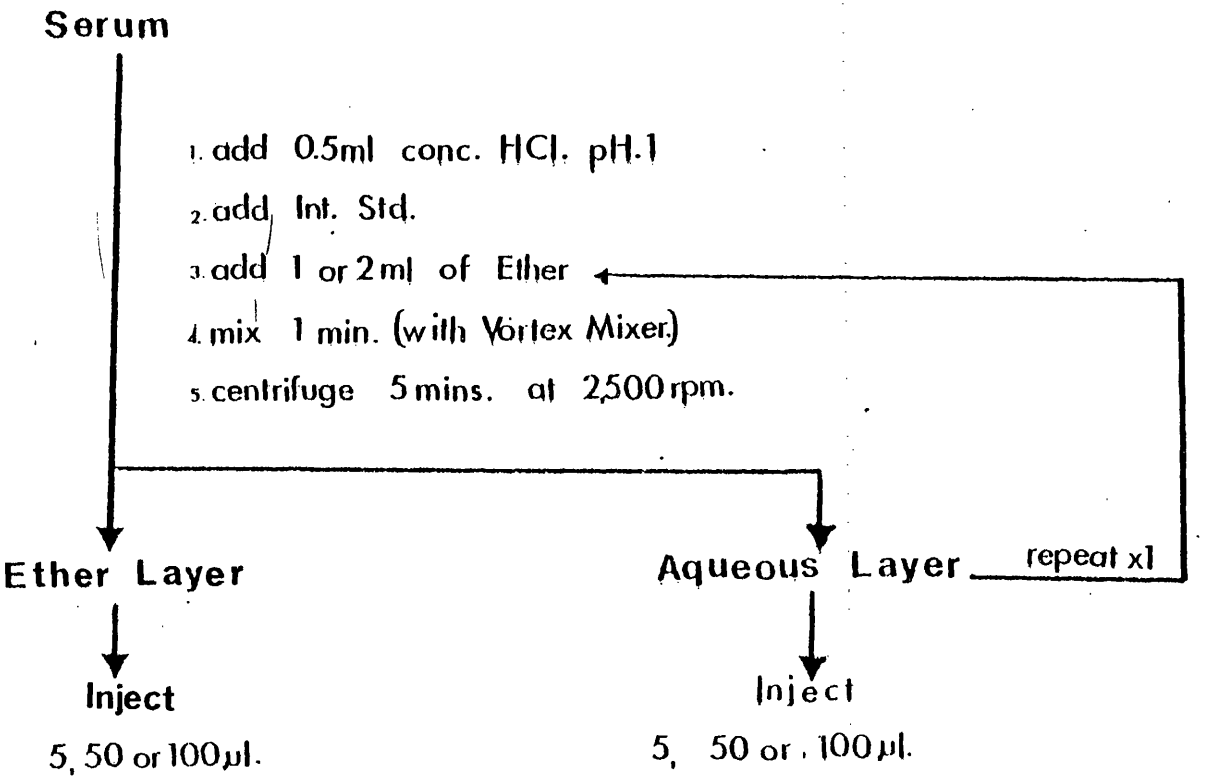


FIGURE 5.1B

Extraction of Drugs from Urine.

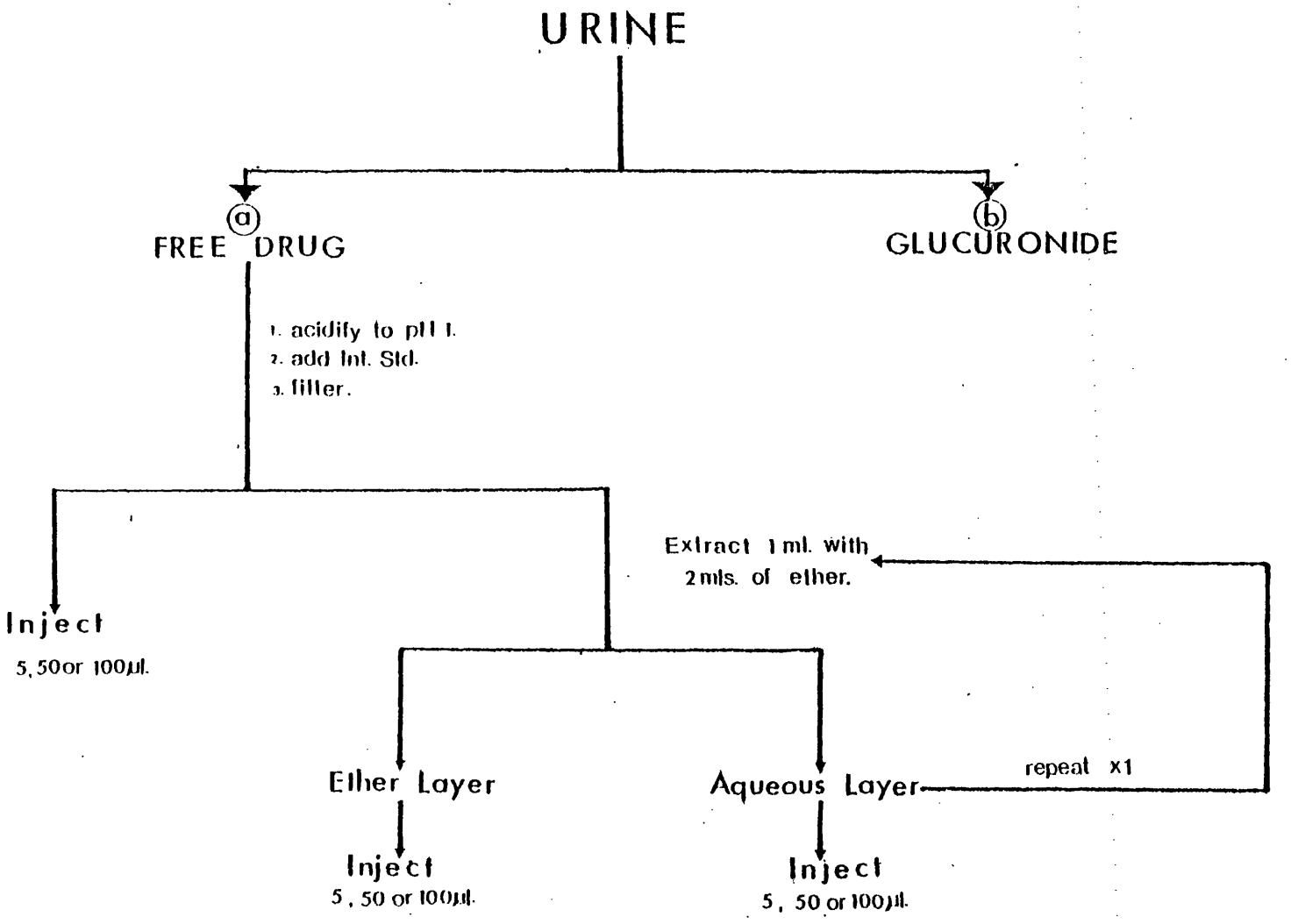


TABLE 5.1 HPLC Conditions For the Agents.

Agents	% Methanol	K′ Values	Detection (λ nm)	Detection Limit(ng)	Internal Standard	Extraction Efficiency			
						Urine Analysis		Plasma Analysis	
						% Rec. n 15	±% Coeff. of Var	%Rec. n 15	±% C.V
1. Oxyphenbutazone	35	6.75	245	5.00	Ketoprofen	99.89	2.5	100.1	1.1
2. Alclofenac	35	5.96	278	50.00	Ketoprofen	97.0	4.5	95.5	3.5
3. Ketoprofen	35	8.50	260	2.5	Oxyphenbutazone	101.1	1.5	106.6	2.1
4. Naproxen	35	10.27	245	2.5	"	99.43	2.7	104.9	4.4
5. Fenoprofen	45	5.50	272	25.0	Indomethacin	101.11	4.5	107.8	9.6
6. Phenylbutazone	45	6.40	254	2.0	"	101.1	1.8	101.8	1.1
7. Sulindac	45	7.25	288	2.5	Naproxen	102.67	1.1	101.1	2.0
8. Indomethacin	45	8.33	260	5.00	Phenylbutazone	104.50	5.8	102.8	3.0
9. Diflunisal	50	6.25	254	2.5	Naproxen	100.87	3.2	98.7	4.3
10. Benoxaprofen	50	8.33	300	2.5	Indomethacin	100.90	4.8	102.6	3.5

5.1.2.3 Evaluation of Extraction Efficiency from Plasma and Urine.

Drug and internal standard were added to control plasma or urine to produce a series of solutions containing 1.0 to 80µg/ml of drug. The solutions were then extracted as described in figure 5.1A and B, chromatographed and the results compared with calibrations obtained using mobile phase only, to give the extraction efficiency, Table 5.1.

The slopes of the calibrations may also be used to provide a % recovery, such that

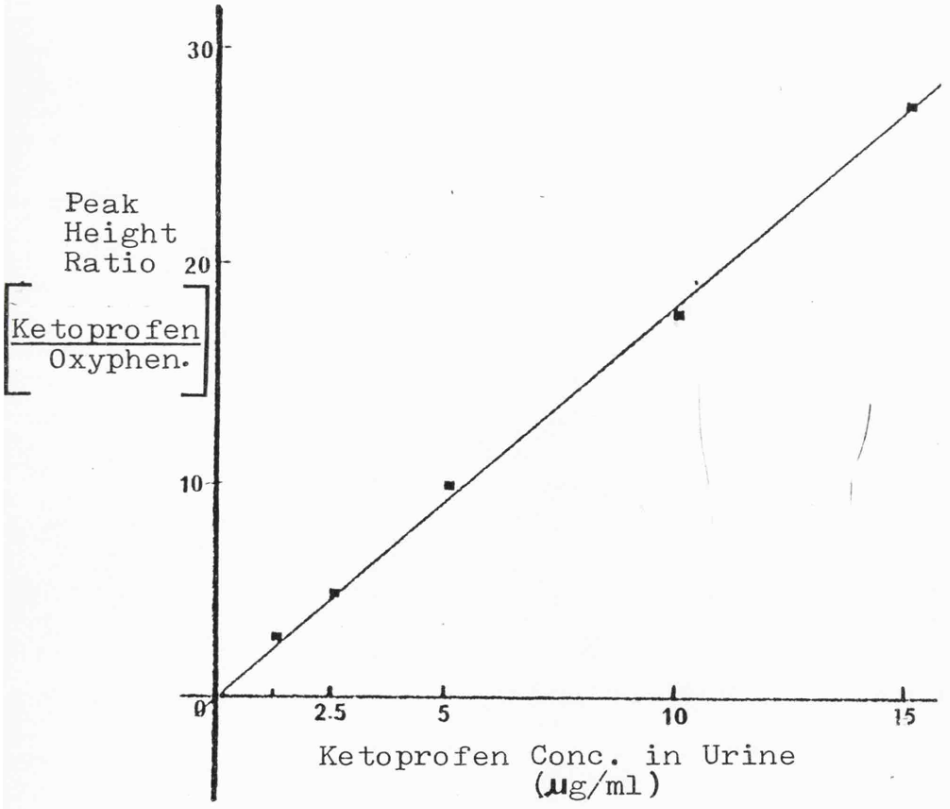
$$\% \text{ Recovery} = \left[\frac{\text{Calculated Slope of Solute Concentration Extracted from Plasma or Urine}}{\text{Calculated Slope of Solute Concentration in the Mobile Phase}} \right] \times 100$$

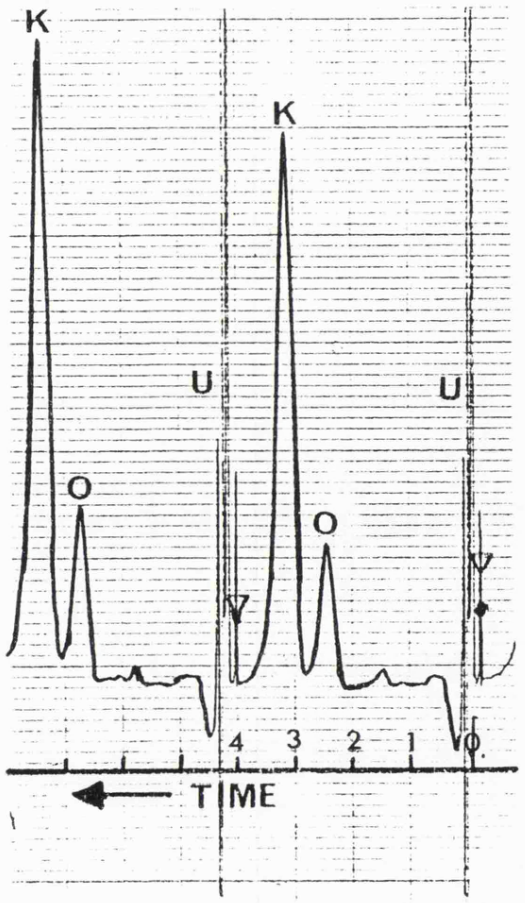
(See Appendix 1.)

With either method, the percentage recovery rates have been found to be greater than 95% for each drug. The assay procedures from urine without extraction and from plasma supernatant were found to be very reproducible. Extraction might be needed in order to improve the life and efficiency of the column when used routinely, but when the solute concentrations are expected to be low, direct injection is advised.

Some of the typical calibration plots and chromatograms of the assay obtained from agents in plasma and urine are illustrated in figures 5.2 to 5.5.

FIGURE 5.2 Assay of Ketoprofen in Urine.





Column - Spherisorb 5-ODS (50x4.6mm id)

Eluent - MeOH:Ac OH (pH 3.0) (35:65)

UV Detection - 260nm

U - Urine Peak

O - Oxyphenbutazone (Internal Standard)

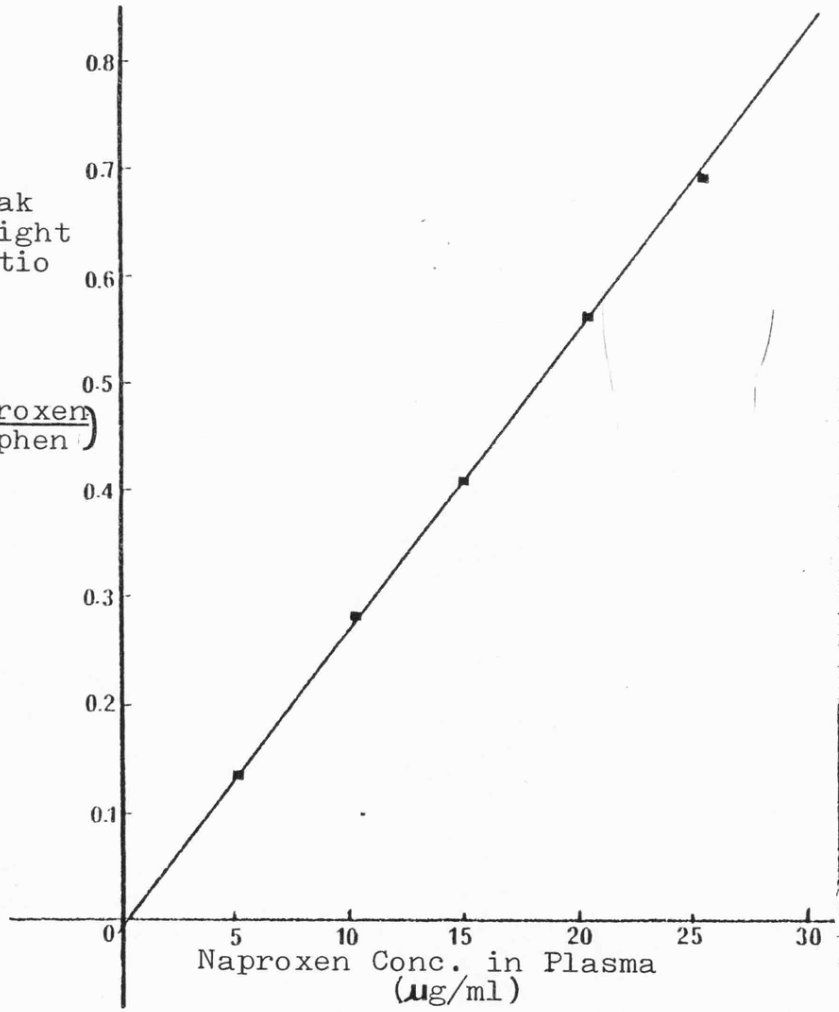
K - Ketoprofen

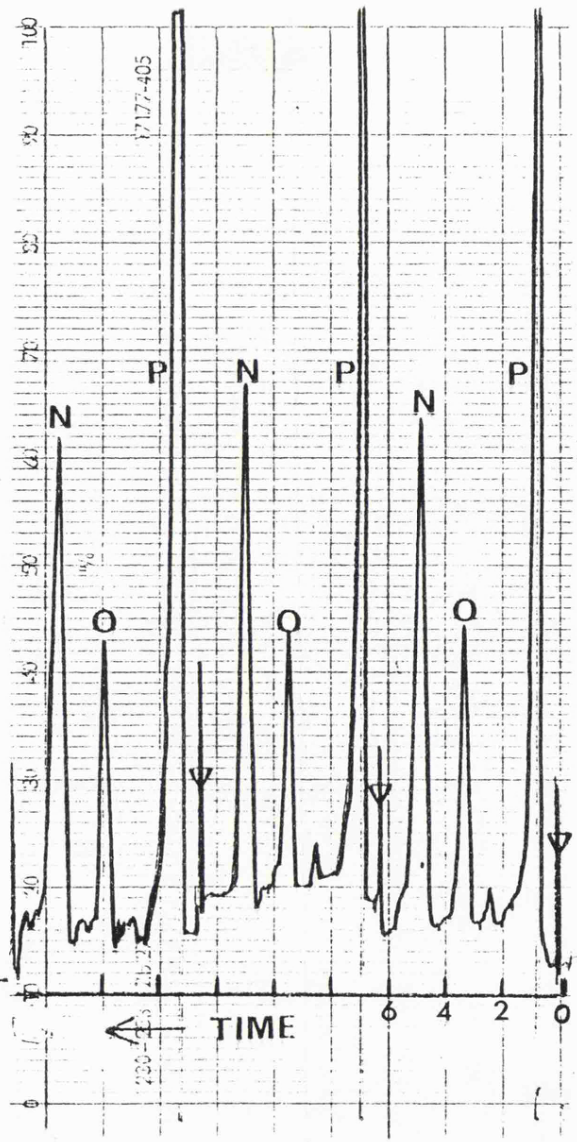
Analysis Time - 4 mins.

Corr. Coeff. = 0.9995

Limit of Error
95% Confidence Limit = ±0.062µg/ml

FIGURE 5.3 Assay of Naproxen in Plasma





Column - Spherisorb 5-ODS (50x4.6mm id)

Eluent - MeOH-AcOH (pH 3.) (35:65)

UV Detection - 245nm.

P - Plasma Peak

O - Oxyphenbutazone

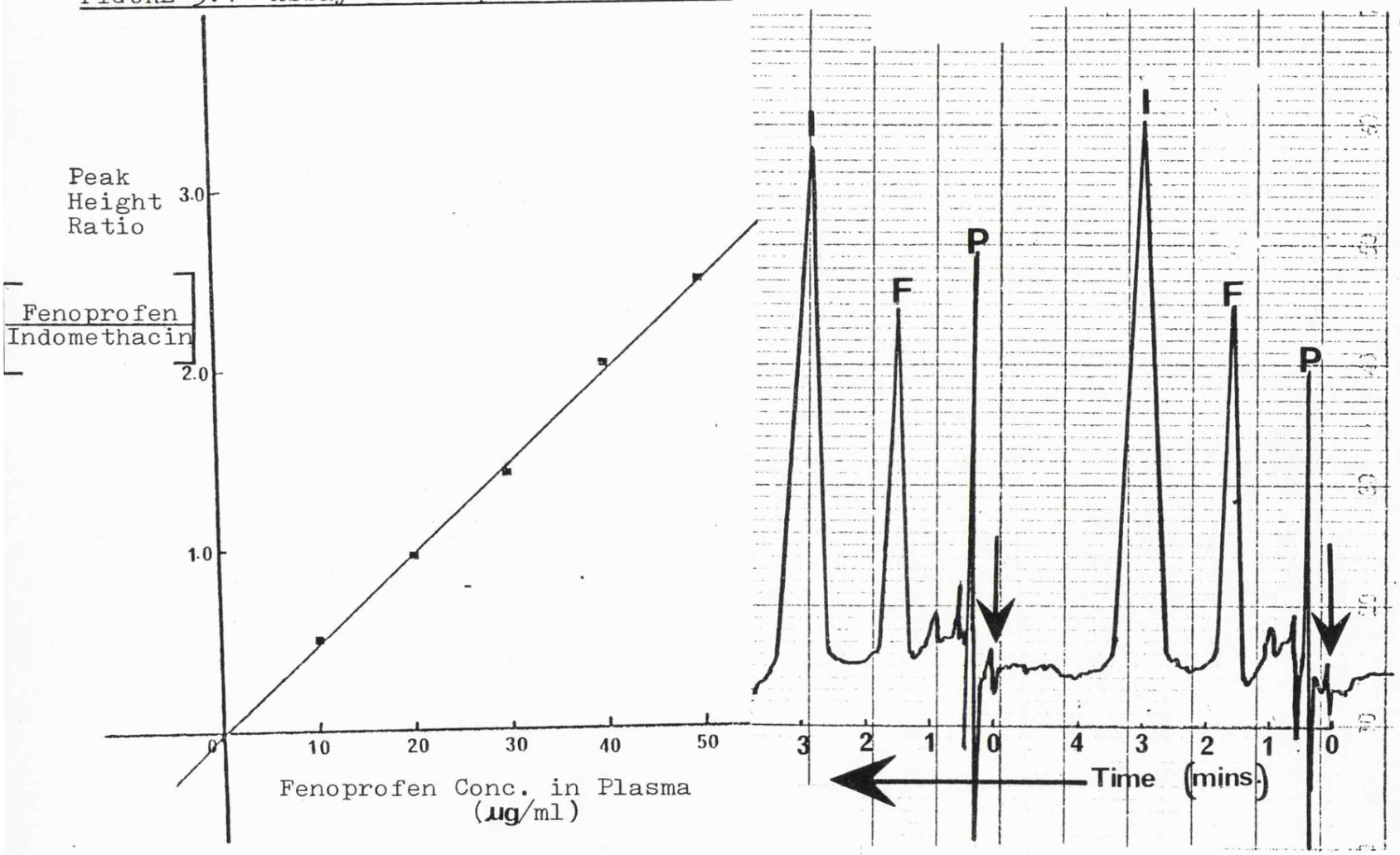
N - Naproxen (internal standard)

Analysis Time - 6 mins.

Corr. Coeff. = 0.998

Limit of Error (95% Confidence Limit) = ±0.016µg/

FIGURE 5.4 Assay of Fenoprofen in Plasma.



Column - Spherisorb (50x4.6mm. id)

Eluent - MeOH-AcOH (pH3) (45:55)

UV Detection - 272nm

P - Plasma Peak

F - Fenoprofen

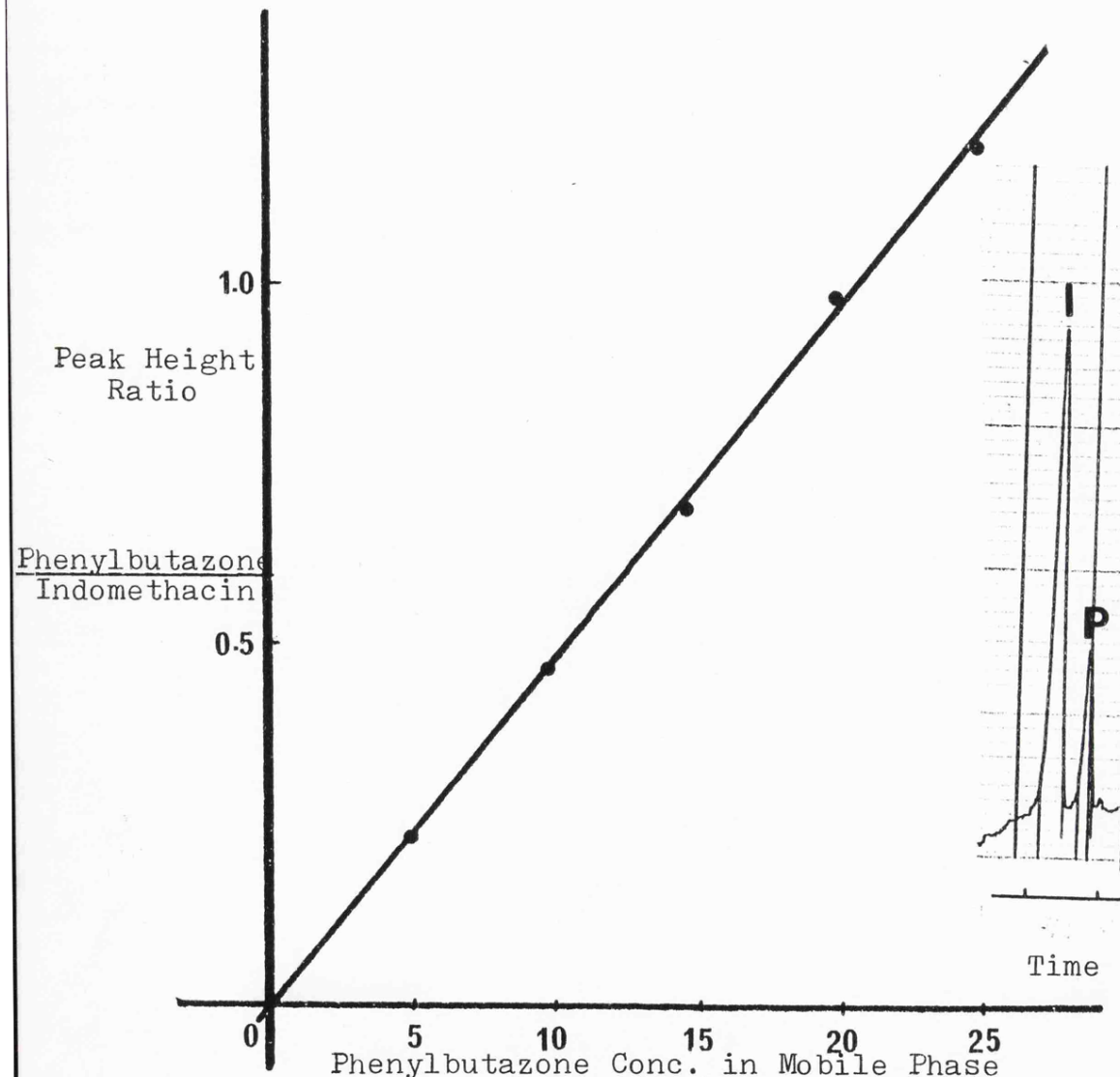
I - Indomethacin (internal standard)

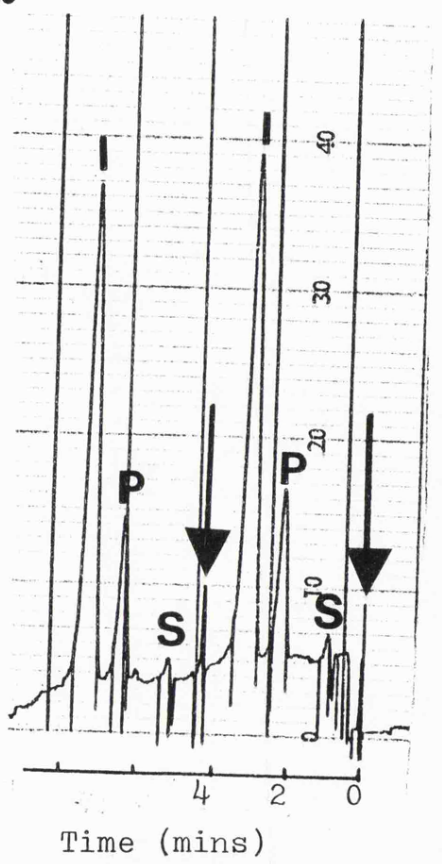
Analysis Time - 4 mins.

Corr. Coeff. - 0.998

Limit of Error - ±0.082 µg/ml (95% Confidence Limit)

Figure 5.5 Assay of Phenylbutazone in Mobile Phase.





Column - Spherisorb 5-ODS (50x4.6mm id.)

Eluent - MeOH:AcOH (pH 3.0) 45:55

Detection - 254nm.

S - Solvent Front

P - Phenylbutazone

I - Indomethacin (internal standard)

Analysis Time - 2 mins.

Corr. Coeff.(r) - 0.999

Limit of Error
95% Confidence Limit = ±0.025µg/ml

5.1.3 Further Applications.

1. This procedure can be used to determine these drugs in other biological fluids. The recovery of the drugs from "spiked" milk and saliva had also been obtained, although of low practical clinical significance.

2. Clinically:-

(i) These drugs are administered chronically and because they are weak acids, their main side effect is gastric ulceration which can impair absorption of the drug. This can be checked by monitoring blood levels, which also provides a check on patient compliance.

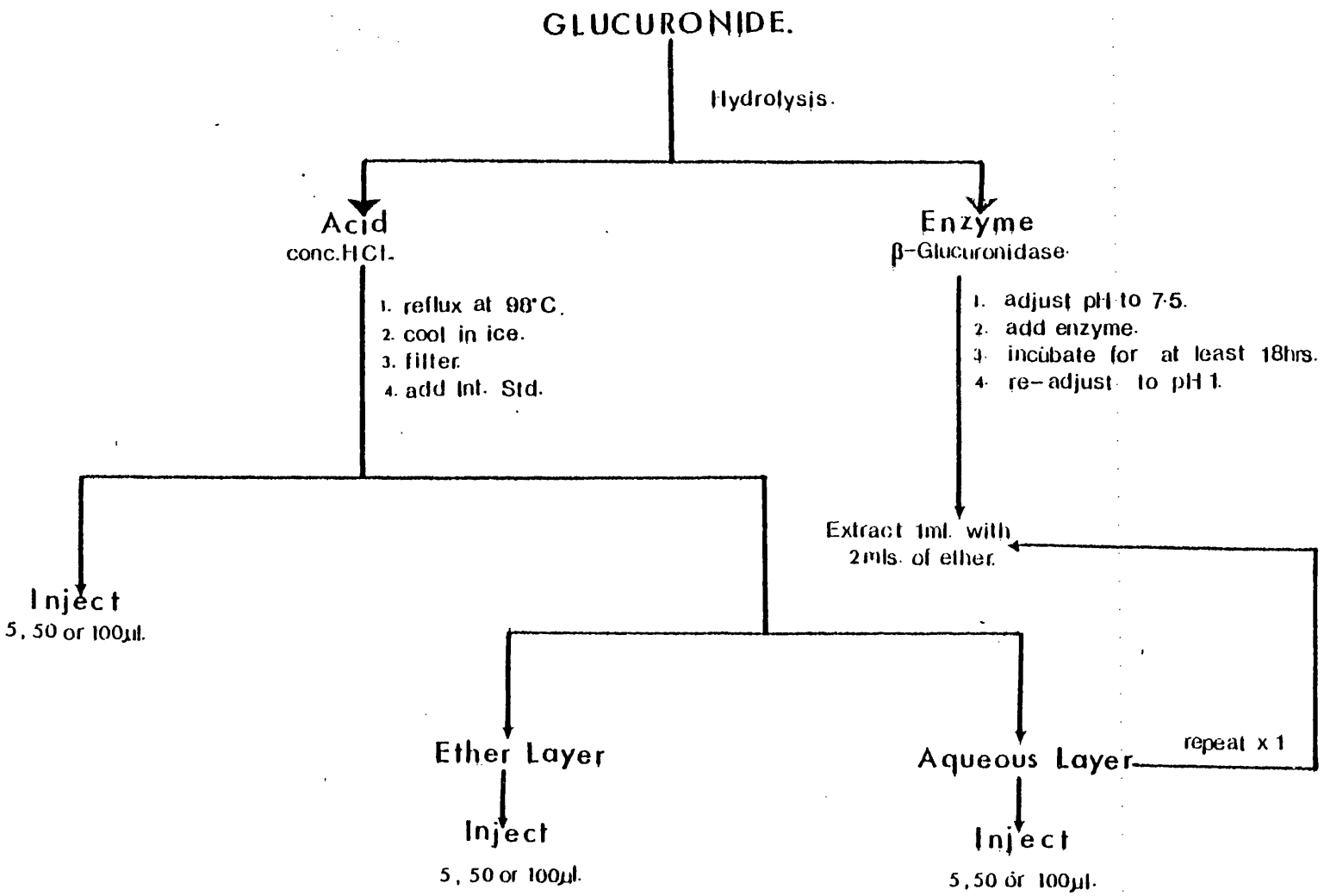
(ii) Most of these drugs are excreted largely as their glucuronide conjugates. The conjugated drug is usually analysed by first converting the conjugate back to free drug by hydrolysis to provide a measure of the total drug excreted in the urine. Hydrolysis was achieved by (figure 5.6) either

(a) addition of 5ml of 6N HCl to 5ml of urine and refluxing for about 30 mins. provided the drug is stable, or

(b) incubating 2ml of urine adjusted to pH 7.5 with phosphate buffer, with 1ml of β-glucuronidase enzyme at 37°C for at least 18 hours.

The glucuronide conjugates of these drugs elute at the solvent front because they are very polar making it difficult for identification and quantification. An

FIGURE 5.6 **Extraction of Drugs from Glucuronide Conjugate.**



attempt to analyse the glucuronide conjugates of ketoprofen and benoxaprofen to establish a procedure for these drug conjugates is described in Chapter 5.3.

(iii) This chromatographic method also provides a routine procedure for the study of future drugs in this group in body fluids. It has been directly employed for the study and clinical trial of a novel anti-inflammatory agent, benoxaprofen, conducted at Royal National Hospital for Rheumatic Diseases in Bath. The results, from the study of 10 patients on a steady dose of 600mg of benoxaprofen per day, showed an average plasma level of 120 ± 37µg/ml (figure 5.7A and B).

Stability of the Drugs.

The drugs have been found to be stable when stored for over a month at about 5°C. in acidified methanol-water or in acidified plasma or urine when stored frozen at about -11°C.

Analysis Time.

The analytical procedure is rapid to perform, one single extraction is usually required before chromatography which takes about 5 mins.

FIGURE 5.7A **ASSAY OF BENOXAPROFEN IN PLASMA.**

Column: 5-ODS.
(50 x 4.6mm.id.)
Mobile Phase: 45% Methanol
Ac-OH. pH 3.

s – Plasma Peak.
a – Int. Std.
b – Benoxaprofen.

1- 2.5µgml^{-1} in plasma.
2-5.0 ,,
3-10.0 ,,
4-15.0 ,,
5-20.0 ,,

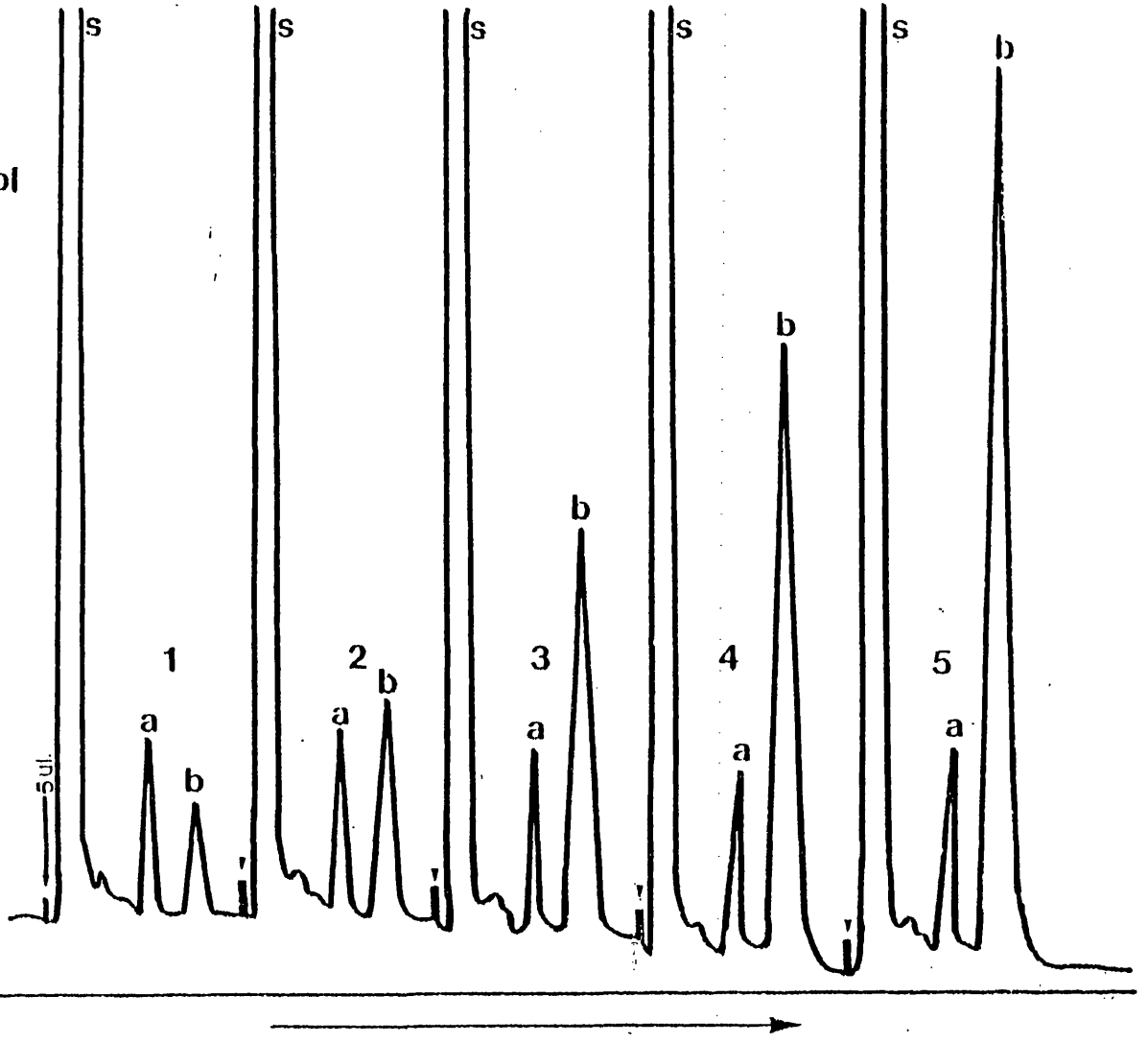
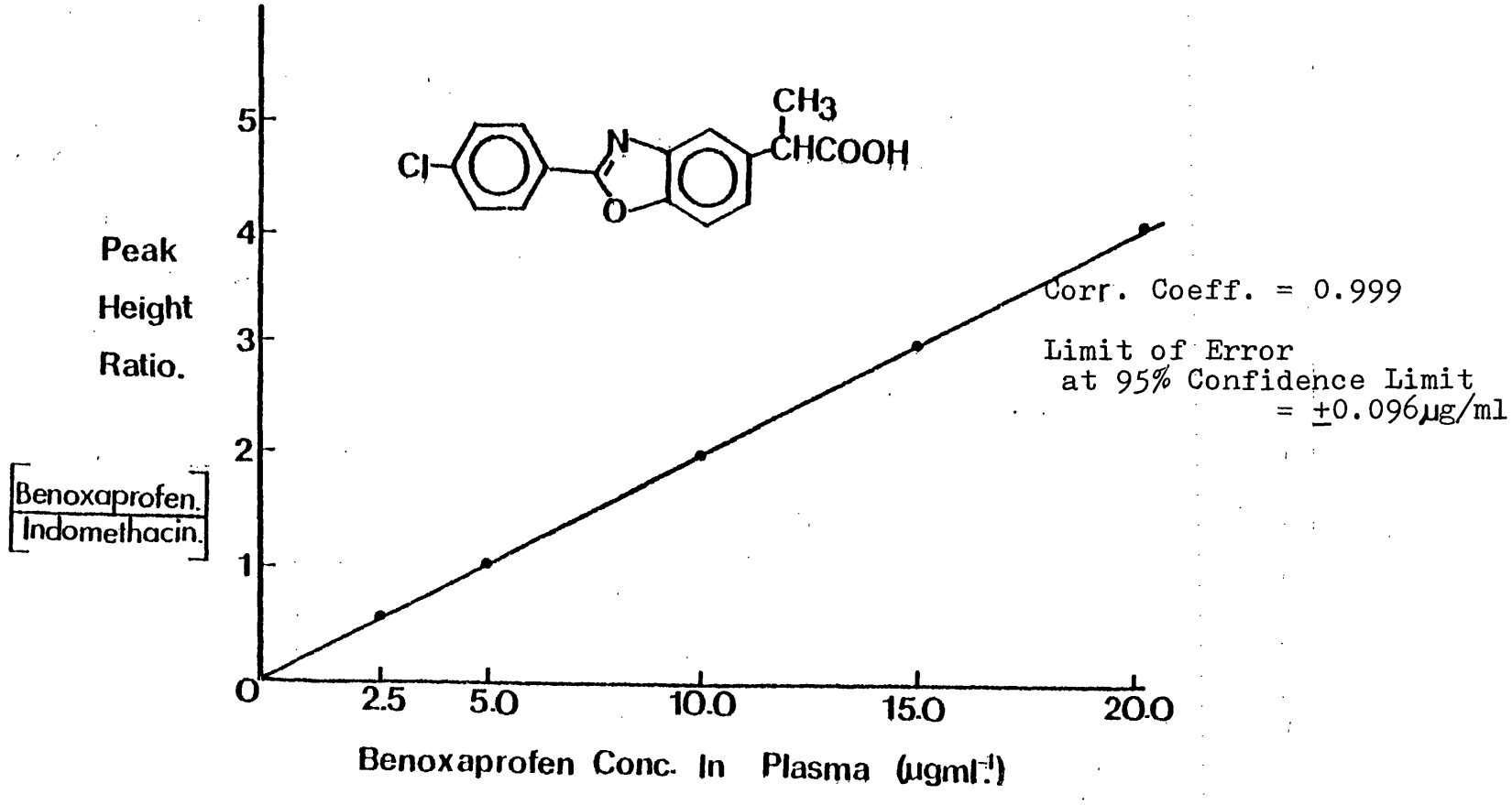


FIGURE 5.7B **ASSAY OF BENOXAPROFEN IN PLASMA.**



5.2 Drugs and Their Metabolites in Body-Fluids.

The study of drug metabolites is important in clinical chemistry because it:-

(i) Provides more information about the nature of the parent drug,

(ii) Explains the duration of action of the drug, and

(iii) Shows the excreted forms of the drug.

The pharmacological activity of drug metabolites is generally different from that of the parent drug, so that the fate of the drug and the rate at which bio-transformation occurs is an important clinical consideration. This is especially so for drugs taken over long periods, and also in the examination of patients suffering from an overdose. It is of special value in Forensic Analysis where there might be total absence of the parent drug due to biotransformation, so that the correct separation and identification of the metabolites found in body fluids would thus lead to the confirmation of the identity of the drug.

Factors to be taken into consideration while developing the method, include high sensitivity and selectivity, ease of sample preparation and the analysis time.

Small quantities of some of the metabolites of indomethacin, naproxen, phenylbutazone and sulindac were obtained from the sources listed in Table 7.1 and so permitted a chromatographic study of these drugs.

Pound, et al. (24,25) have described methods for the simultaneous determination of phenylbutazone and oxyphenbutazone which have been discussed in Chapter 1.4. Very recently, Wan and Matin (69) described a GLC method involving butylation which differentiated between naproxen and its 6-desmethyl metabolite and were quantified simultaneously in urine. No other HPLC procedures have been described for any of these drugs with their metabolites.

Reversed phase chromatography with aqueous acidic methanol was chosen because the metabolites are usually more polar than the parent drug and so elute sooner. Total analysis time was therefore defineable, and the retention range available for the metabolites was limited. This is a major advantage over the HPLC method described by Pound (25). In the absence of standards, this was also an advantage because it offered the ability to determine the end of the analysis and to suspect any major peak eluting before the parent compound as the metabolite.

5.2.1 Effect of Changing Eluent Strength on the Capacity Factor (K′) and the Selectivity (α) of Drugs and their Metabolites.

In order to determine the optimum composition of the mobile phase for the four drugs and their metabolites, the effect of adjusting the methanol composition on retention and selectivity was studied, using the conditions previously described for ionic suppression. The results are expressed in Table 5.2 and illustrated in figures 5.8 A-D, Chromatograms of:-

(i) Analysis of indomethacin and its metabolites using phenylbutazone as the internal standard, fig. 5.9 and

(ii) Analysis of naproxen and its metabolite with oxyphenbutazone as the internal standard, fig. 5.10.

There was a good correlation between the elution order of the drugs and their metabolites and their relative hydrophobicities, as predicted. Also, the relationship between the log methanol concentration of the mobile phase and log K′ of the drugs and their metabolites from Table 5.2 produced correlation coefficients of between 0.96 and 0.999. The separation obtained produced reliable quantification in a reasonable analysis time.

The results clearly show that good separations are possible for these drugs and their metabolites. The differences observed in the K′ values between drug and its metabolite are due to changes in drug polarity (eg)

TABLE 5.2 Effect of Changing Eluent Strength on the Capacity Factor (K′) and the Selectivity (α) of Drugs* and Their Metabolites.

Column - Spherisorb 5-ODS (50x4.6mm id.)
Mobile Phase - Methanol:Water, Acetic Acid (pH 2.5)

	Drugs* and Their Metabolites	30% MeOH		40% MeOH		50% MeOH		60% MeOH		70%MeOH	
		K′	α	K′	α	K′	α	K′	α	K′	α
1	Desmethylnaproxen	4.67	4.00	2.00	3.67	1.00	2.33	0.44	2.25	0.00	∞
	Naproxen*	18.67		7.33		2.33		1.00		0.50	
2	Oxyphenbutazone	13.00	-	4.33	3.23	1.33	3.00	0.67	2.00	0.00	∞
	Phenylbutazone*			14.00		4.00		1.33		0.60	
3	Sulphone	-	-	11.33	1.85	3.00	1.33	1.00	1.33	0.33	1.00
	Sulindac*	-		21.00	-	4.00	5.58	1.33	4.25	0.33	4.00
	Sulphide	-				22.33		5.67		1.33	
4	DeschloroIndomethacin	-	-	1.33	5.75	0.67	3.50	0.33	3.00	0.00	∞
	DesmethylIndomethacin	-		7.67	4.65	2.33	2.43	1.00	2.00	0.33	2.00
	Indomethacin*	-		35.67		5.67		2.00		0.67	

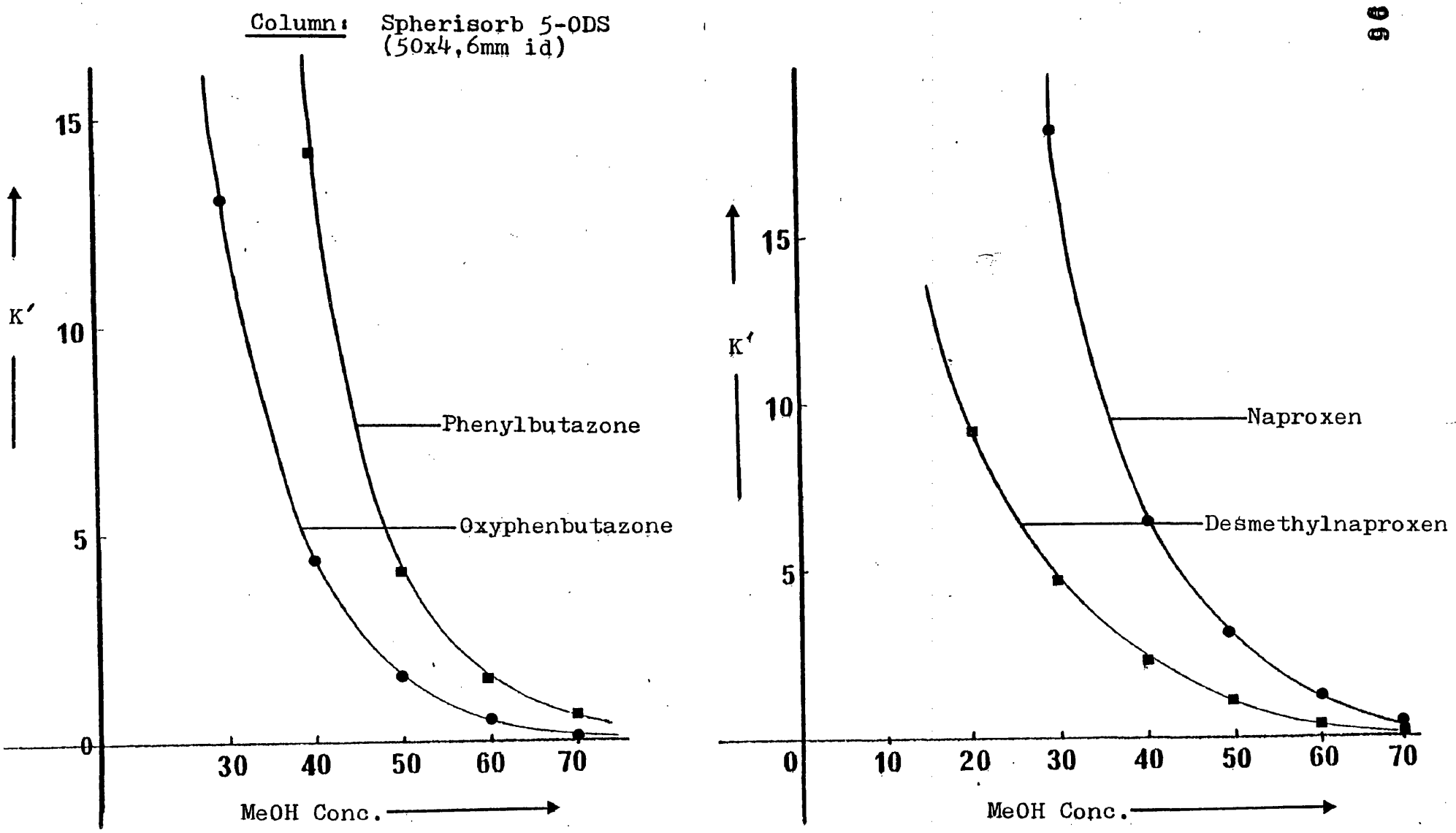


FIGURE 5.8A & B Effect of Eluent Strength (MeOH Conc.) on Capacity Factor (K′) of Drugs and Their Metabolites.

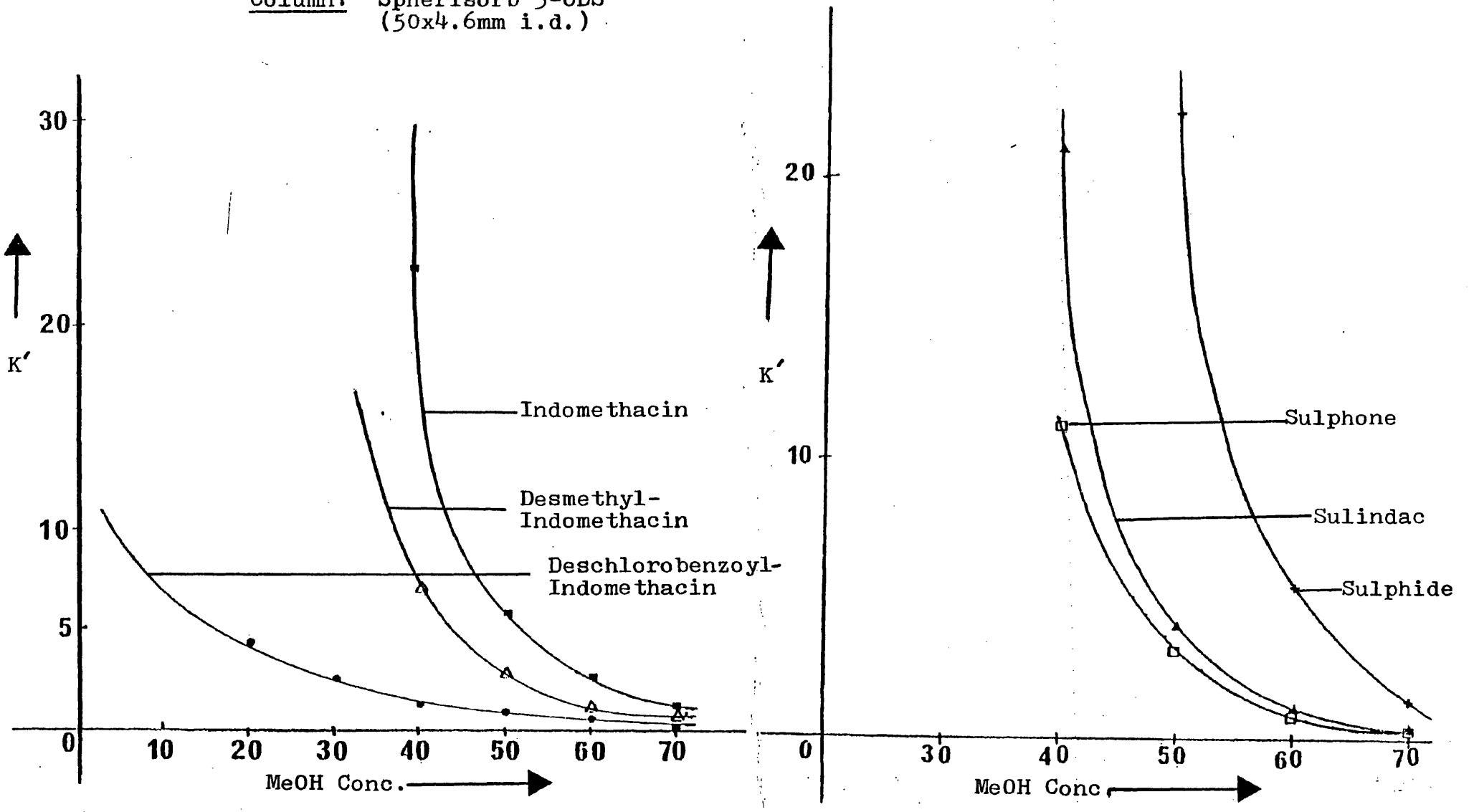
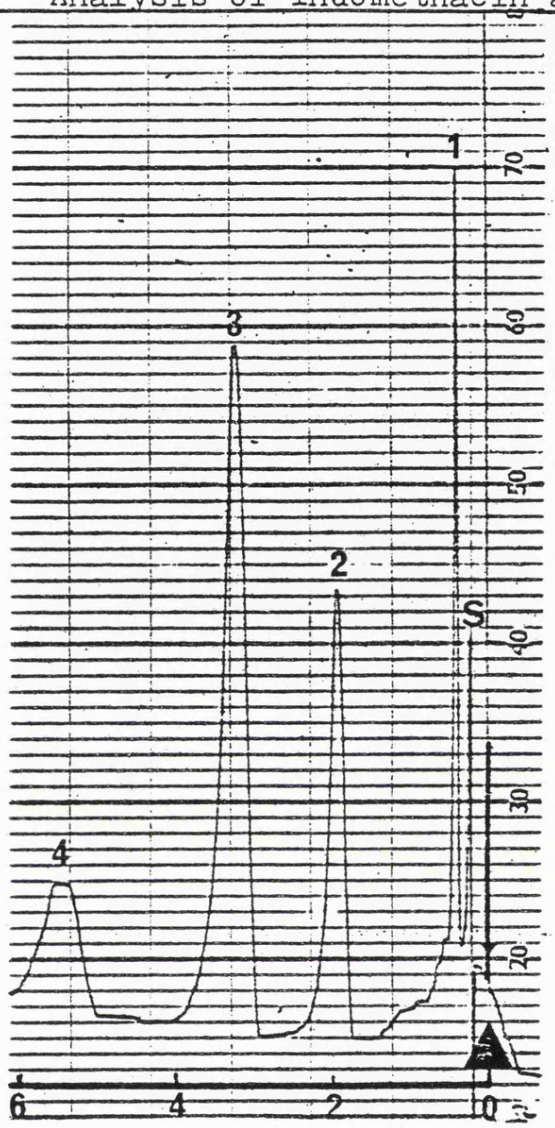


FIGURE 5.8C & D Effect of Eluent Strength (MeOH Conc.) on Capacity Factor (K′) of Drugs and Their Metabolites.

FIGURE 5.9 Analysis of Indomethacin and Its Metabolites.



Column: Spherisorb 5-ODS. (50x4.6mn id.)

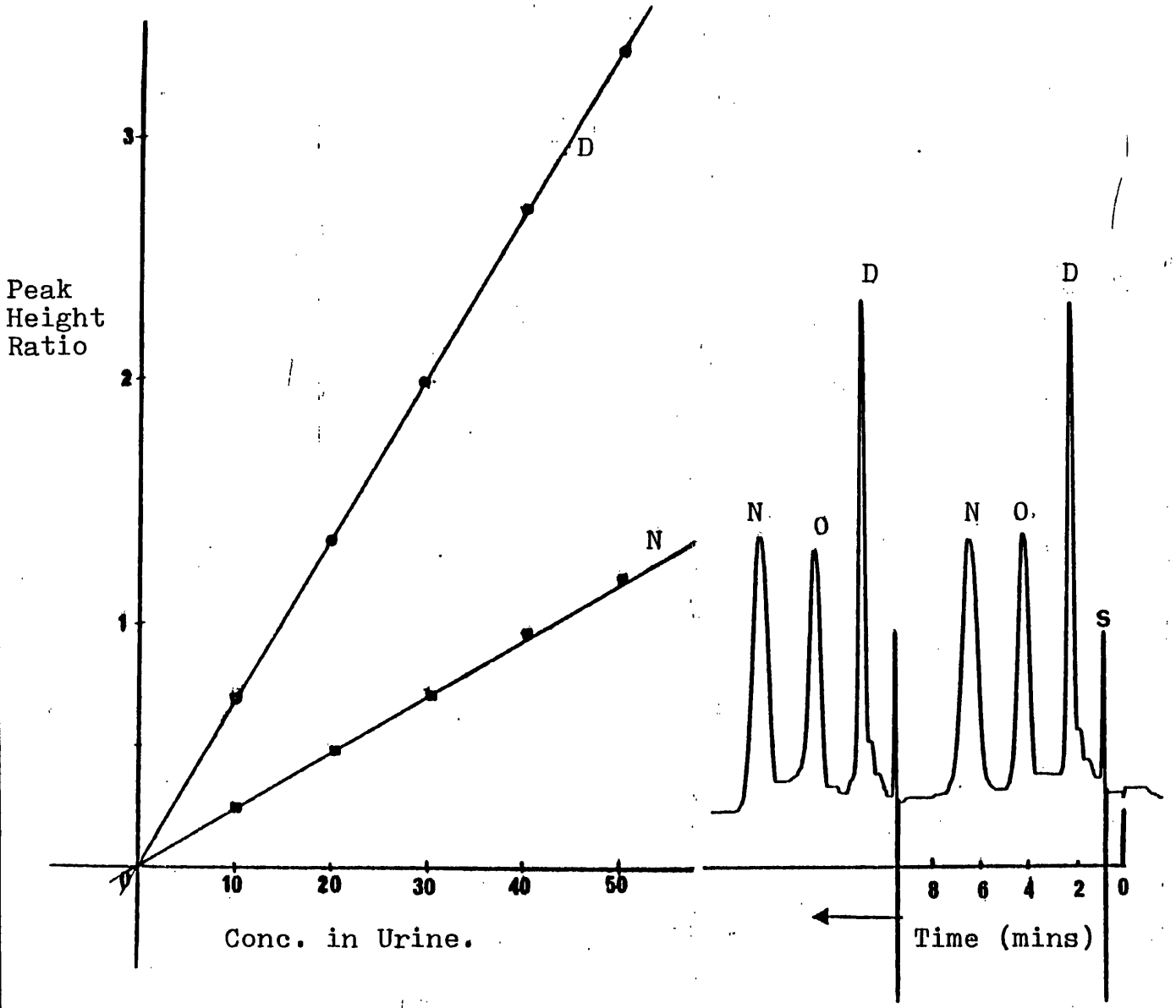
Mobile Phase: MeOH:H_2OAc. (40:60) pH 2.5

Detection: 254nm.

Int. Std.: Phenylbutazone

Peaks: S - Solvent
1 - Deschloro benzoylindomethacin.
2 - Desmethylindomethacin.
3 - Phenylbutazone.
4 - Indomethacin.

FIGURE 5.10 Analysis of Naproxen and Its Metabolite in Urine.



Column - Spherisorb 5-ODS (50 x 4.6mm id)

Eluent - MeOH - AcOH (pH 2.5) (30:70)

UV Detection - 245nm

Attenuation - 0.05 Aufs.

S - Urine front.

D - Desmethylnaproxen.

O - Oxyphenbutazone (internal standard)

N - Naproxen.

(1) the loss of a methoxy [-OCH_3] group and a gain of a [-OH] group in naproxen and indomethacin to give more polar desmethylnaproxen and desmethylindomethacin, respectively.

(2) the hydroxylation of one phenyl group of phenylbutazone to form a more polar oxyphenbutazone.

(3) the loss of chlorobenzoyl group in indomethacin to form a more polar deschlorobenzoyl indomethacin, and

(4) the metabolism of sulphoxide to produce a more polar sulphone derivative of sulindac.

The reversed phase system has thus permitted these polar metabolites to elute before the parent drugs whereas they are longer retained in adsorption chromatography or accumulate on the column, gradually degrading its performance.

5.2.2 Determination of Sulindac and its Sulphone and Sulphide Metabolites in Plasma and Urine

One exception to the general rule that metabolites are less hydrophobic than the parent drug, is provided by the sulphide metabolite of sulindac. As a consequence this metabolite is excessively retained, leading to a reduced peak size and sensitivity of detection, making the method unsuitable for quantitative analysis.

This problem was solved by using an amino-bonded phase, that possesses the polar functional group NH_2 bonded to the hydrocarbon chain. This makes the phase less hydrophobic than the octadecyl phase and so shortens retention due to hydrophobic interactions.

Optimum retention of sulindac and its metabolites on this column is brought about by careful changes in the isopropanol-acetic acid content of the mobile phase. Decrease in acetonitrile content of the eluent brings about early elution of the compounds while increase causes retention.

5.2.2.1 Assay Procedure.

The chromatographic condition was set up with the filtered mobile phase to establish a steady base line on the recorder.

To 1ml of plasma in a 10ml glass centrifuge bottle, 1ml of conc. HCl was added to precipitate the proteins, then the drug and its metabolites were extracted in 5ml of ether. 5 or 25µl of the ether extract was then injected onto the column.

Calibration.

Calibration samples were prepared by measuring 50µl of suitable sulindac, sulphone and sulphide solutions into 5ml tubes of plasma or urine each with 500ng naproxen, as

internal standard to make 5 samples containing 4, 8, 12, 16 and 20µg/ml respectively of the compounds. (figure 5.10)

5.2.2.2 Results.

Calibration graphs of peak height ratios of sulindac to naproxen vs. sulindac concentration; sulphone to naproxen vs. sulphone concentration; and sulphide to naproxen vs.sulphide concentration were constructed. The graphs were straight lines passing through the origin (with correlation coefficient 0.999) (Figure 5.11)

Reproducibility.

Samples of spiked urine and plasma from healthy humans were analysed. Table 5.3 shows that this procedure permits the quantitative assay of sulindac and its metabolites down to 4µg/ml, though when a higher sensitivity is required, larger sample volume, eg. 25, 50 or 100µl of the extract, could be injected. The absolute detectable concentration was 2.5ng. at 0.2AUFS. The yields ±% Coefficient of variation of extraction from plasma or urine are

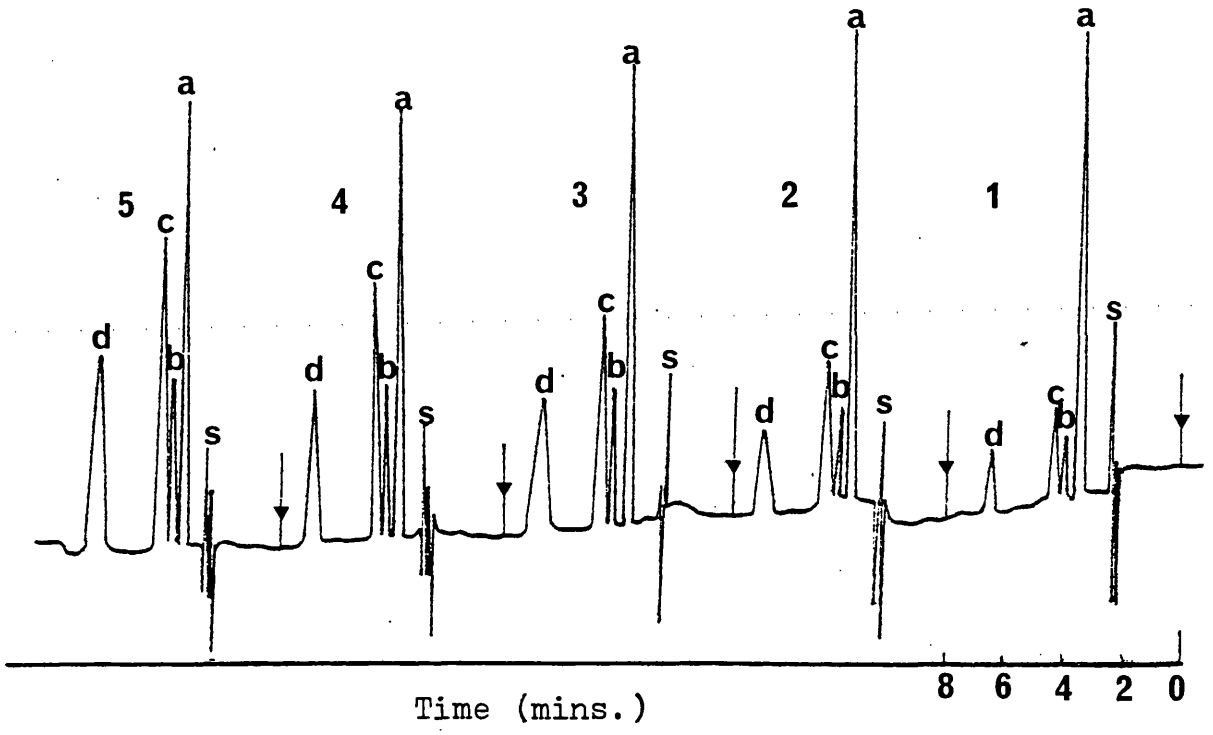
95 ±6.0% for sulindac,

99 ±8% for sulphide (n = 17)

98 ±6% for sulphone.

The metabolites were found to be stable in the solution of the mobile phase at pH 2.5. No decrease in the drug and its metabolites was observed in plasma and urine when stored frozen for a month.

FIGURE 5.10 Assay of Sulindac and Its Metabolites in Plasma.



Column: Spherisorb 5-NH_2 (250x4.5mm id)

Mobile Phase: Acetonitrile:Isopropanol:Acetic Acid. (97:3:1)

Detection : 288nm

Injection : 5µl of Ether Extract of Plasma.

s - Ether Peak.

a - Int. Std. (Naproxen)

b - Sulphide.

c - Sulphone.

d - Sulindac

1 - 4µg/ml in plasma.

2 - 8 " "

3 - 12 " "

4 - 16 " "

5 - 20 " "

FIGURE 5.11 Calibration Plot of Sulindac and its Metabolites.

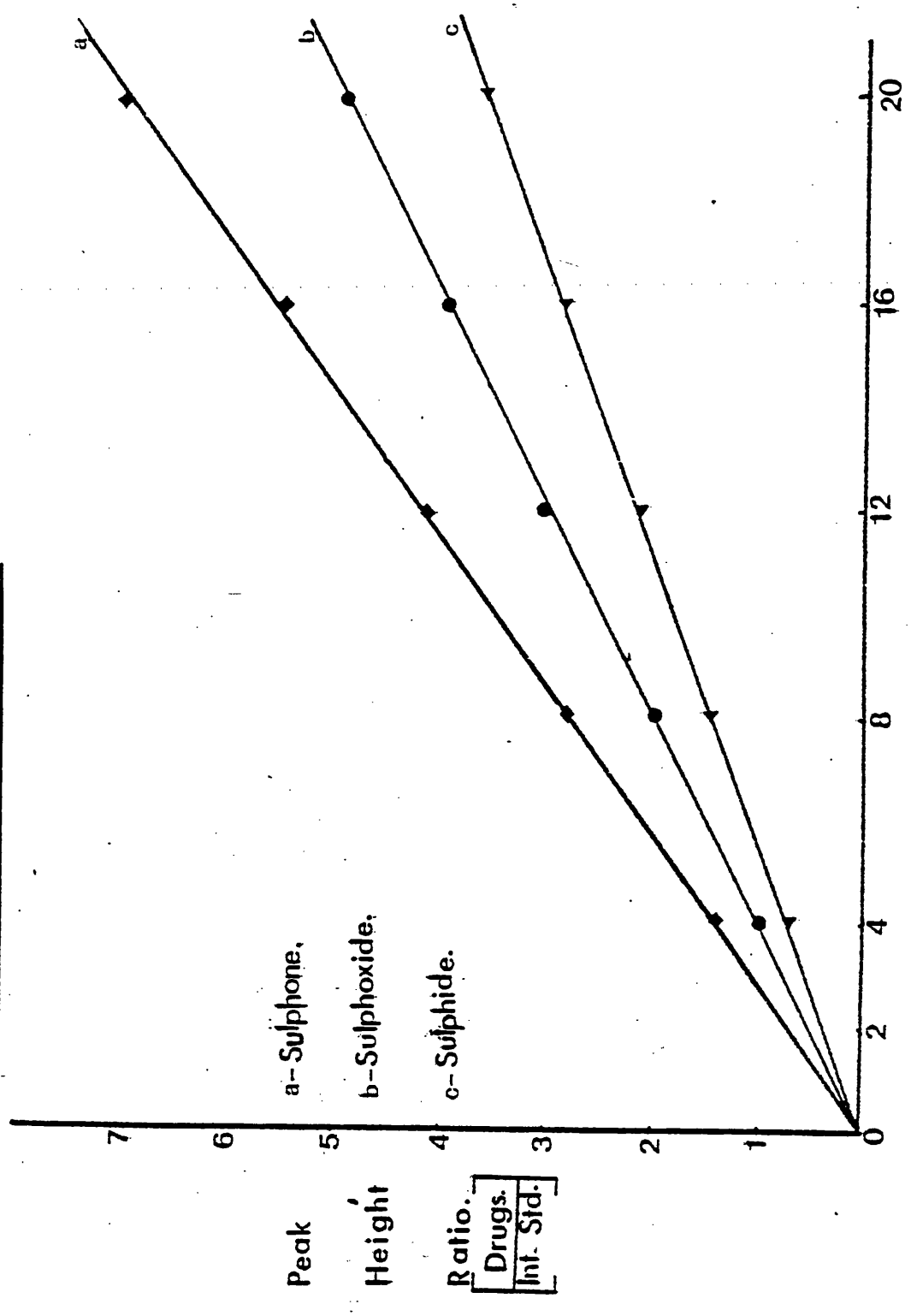


TABLE 5.3 Reproducibility and Accuracy of the Assay of Sulindac and Its Metabolites in Human Plasma and Urine.

5µl of Ether Extract Injected.

Compound	Sample (1ml)	Amount Added (µg/ml)	Average % Recovery of 4 Assays	±% Coefficient of Variation
Sulindac	Plasma	4	94.16	0.65
		8	98.58	2.20
		12	93.21	0.39
		16	93.51	0.80
		20	97.80	2.05
	Urine	4	112.37	1.00
		8	96.09	2.63
		12	93.19	2.01
		16	97.59	0.72
		20	97.29	2.40
Sulphide	Plasma	4	99.27	0.75
		8	95.75	4.89
		12	97.11	1.77
		16	103.65	0.26
		20	101.45	2.68
	Urine	4	132.72	3.48
		8	101.39	2.25
		12	100.03	1.06
		16	100.13	2.14
		20	97.24	2.30
Sulphone	Plasma	4	98.59	1.72
		8	95.50	1.81
		12	98.57	2.17
		16	99.11	0.10
		20	101.06	1.10
	Urine	4	111.30	2.36
		8	96.87	0.90
		12	98.04	1.09
		16	97.59	1.32
		20	99.72	0.9C

Sulindac and its metabolites provide an interesting variation in the study of drugs and their metabolites. Compared to sulindac, the sulphide metabolite has a longer half-life in plasma, and possesses a more potent anti-inflammatory property. Figure 5.12 shows the profile of sulindac and its metabolites in the plasma of a healthy volunteer following an oral dose of 200mg of sulindac. In urine, the sulphone is the major form together with a small amount of free drug.

The sulphide is also more lipophilic than sulindac which means that it will have a larger K' value when chromatographed on a reversed phase column. Typical K' values were 3.0, 4.0 and 22.3 obtained for sulphone, sulindac and sulphide respectively (figure 5.8D).

Further works carried out on the amino-bonded silica material revealed that the stationary phase could be employed also for the separation of naproxen and desmethyl-naproxen. It is not suitable for indomethacin and its metabolites because indomethacin and deschlorobenzoyl-indomethacin could not be sufficiently-well resolved for accurate analysis.

The amino-bonded stationary phase has been applied to analyze the plasma and urine samples collected from a patient during a change over period between sulindac and naproxen therapy (figure 5.13A and B). The

FIGURE 5.12 Profile Study of Concentrations of Sulindac and Its Metabolites in Plasma of a Healthy Volunteer After an Oral Dose of 200mg of Sulindac.

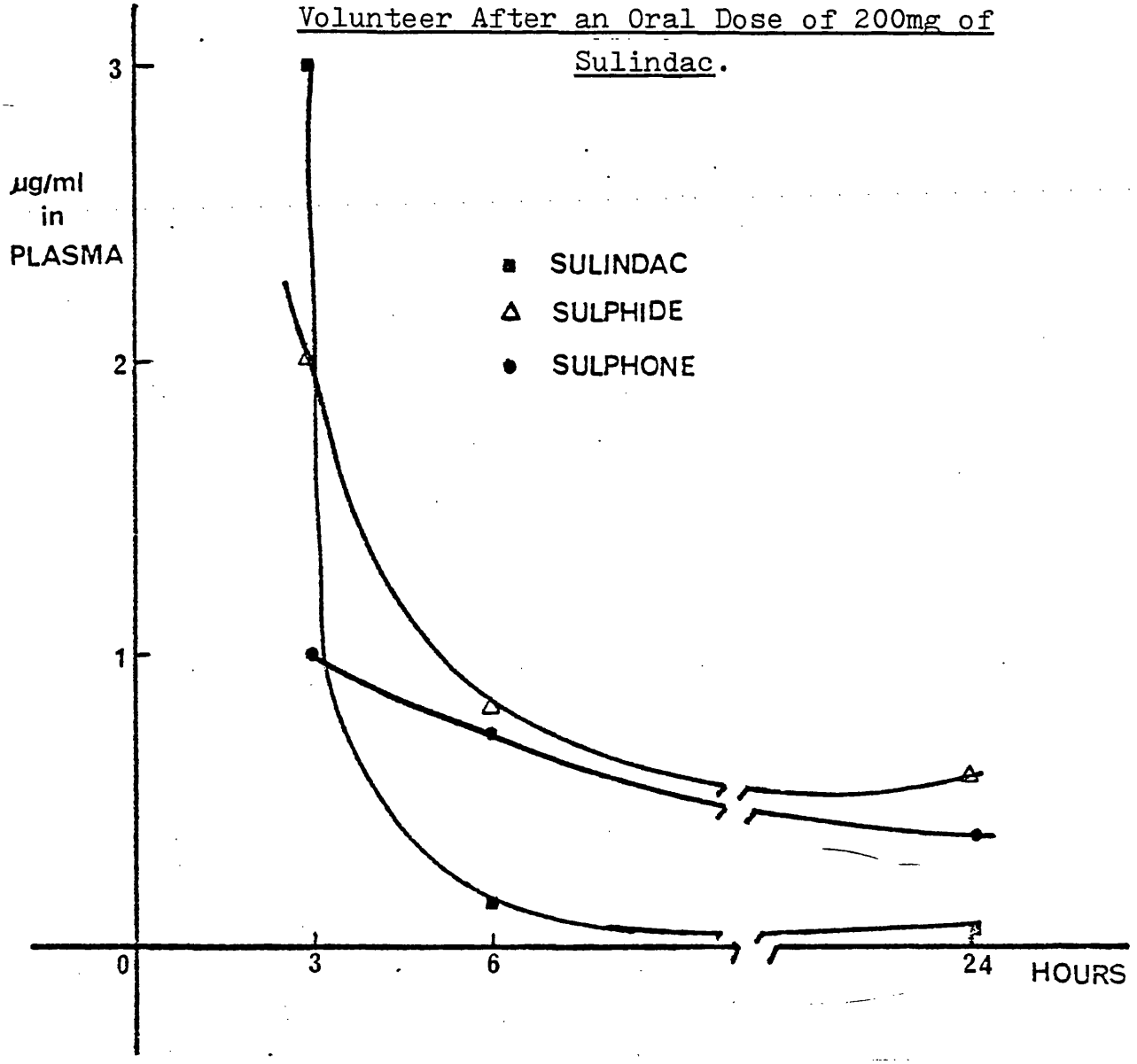
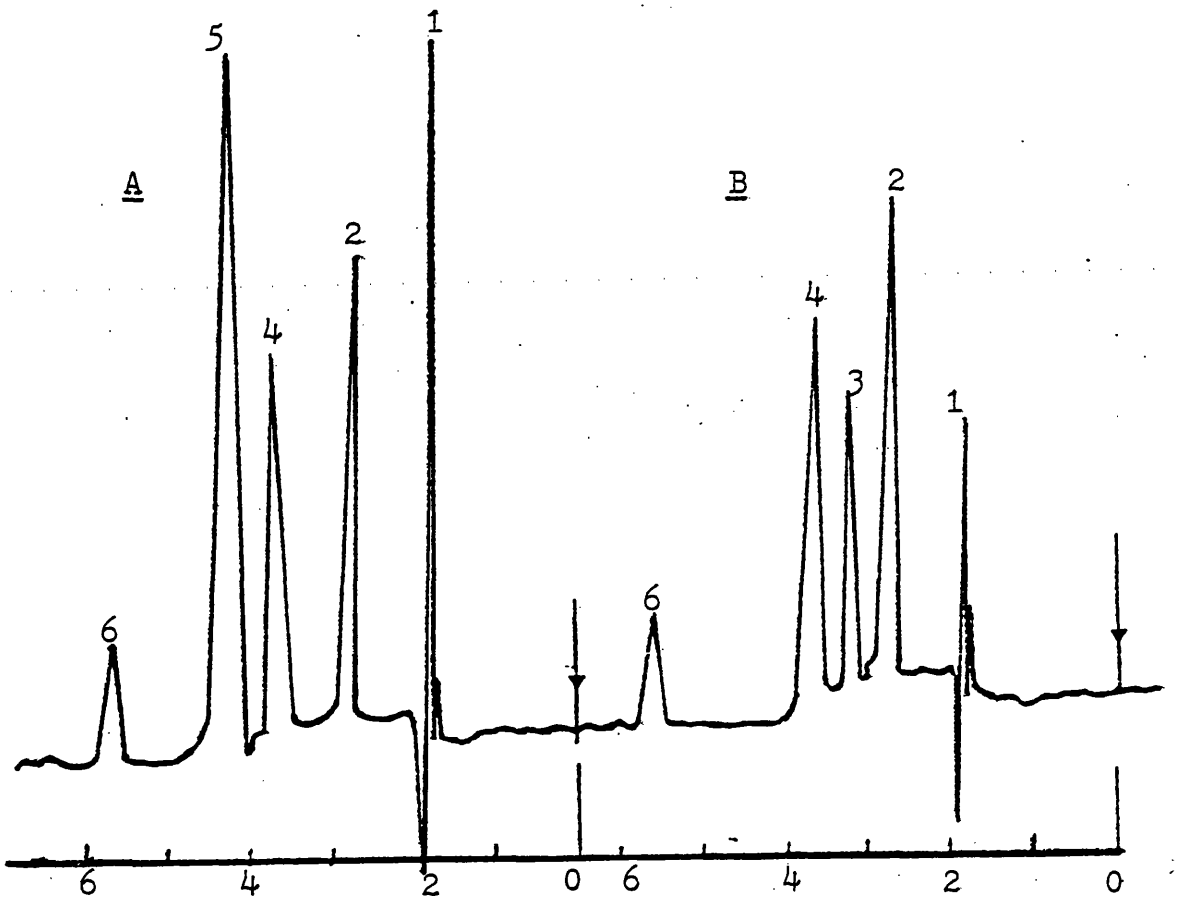


FIGURE 5.13 Clinical Samples of a Patient Receiving Clinoril (Sulindac) and Naproxen Drugs.



A - Sample of Clinical Urine

B - Sample of Clinical Plasma

1 - Organic Solvent Front

2 - Naproxen

3 - Sulphide

4 - Sulphone

5 - Desmethylnaproxen

6 - Sulindac

Column - Spherisorb - 5NH_2
(250x4.6mm id)

Mobile Phase - Acetonitrile:Isopropanol-Acetic Acid.
(96.3.1)

UV Detection - 288nm.

chromatograms obtained illustrate that the metabolites are all resolved, and show the absence of desmethylnaproxen in the clinical plasma sample with the presence of desmethylnaproxen and absence of sulphide metabolite in the urine sample.

The same single extraction procedures described in Chapter 5.1 have been employed and the % recovery has been greater than 98% ±4% coefficient of variation for the drugs and their metabolites. (See Appendix.)

The amino bonded phase packing material is newly introduced and recommended by the manufacturers for polar solutes. It is employed here in a form of straight phase chromatography (figure 2.6). It is weakly polar; the mobile phase is non-aqueous and acidic. The mechanism of retention is a mixture of hydrogen-bonding and hydrophobic interactions. The hydrogen-bonding will also occur with the isopropanol in the mobile phase, therefore an increase in the percentage of isopropanol will decrease retention.

The differences observed in the K' values between the drug and its metabolite are due to changes in drug polarity, eg., naproxen ⟶ desmethylnaproxen, a loss of methoxy ($-OCH_3$) and a gain of -OH which has conferred high polarity necessary for its excretion. The higher K' value for desmethylnaproxen, figure 5.13, further shows that the retention mechanism is predominantly due to polar interactions in the stationary phase.

The major disadvantage in the use of an amino-bonded silica column is the inability to inject the biological fluid directly onto the column. The compounds first have to be extracted into a suitable organic solvent such as ether (figure 5.1A and B). The sensitivity obtained using this column has been found to be considerably higher than using a reversed phase column.

5.2.3.A Comparison of the metabolism of naproxen under different biological conditions.

The study of drug metabolites has been achieved using four different methods:-

(1) Animal liver homogenate preparation,

(2) Dosing healthy rabbits,

(3) From healthy human volunteers given the drugs, and

(4) From clinical samples obtained from patients who were being treated with these drugs.

The first two methods were carried out in the school while three and four were carried out in the Royal National Hospital for Rheumatic Diseases, Bath.

The liver homogenate was prepared by slight modifications of the published methods. (95) The incubation was carried out in unstoppered conical flasks at 37°C. for at least 3 hours. Each incubate contained-

(a) Substrate - the drug 7mg/ml in water - 1ml

(b) Liver homogenate 1gm/ml - 2mls

(c) Ice-cold phosphate buffer pH 7.5 - 3mls

(d) Co-factor solution - 1ml

Total volume per flask - 7mls

The 1ml of the cofactor solution consisted of:-

(a) Glucose-6-phosphate sodium (20 µmoles) 6.0mg

(b) Nicotinamide adenine diphosphate - disodium salt $NaDPNa_2$ (4 µmoles) 3.4mg

(c) Magne sium chloride sol. ($MgCl_2$) (0.1M) 0.2ml

(d) Distilled water

Three sets of experiments were carried out concurrently.

(A) Control homogenate incubate, i.e. no substrate added.

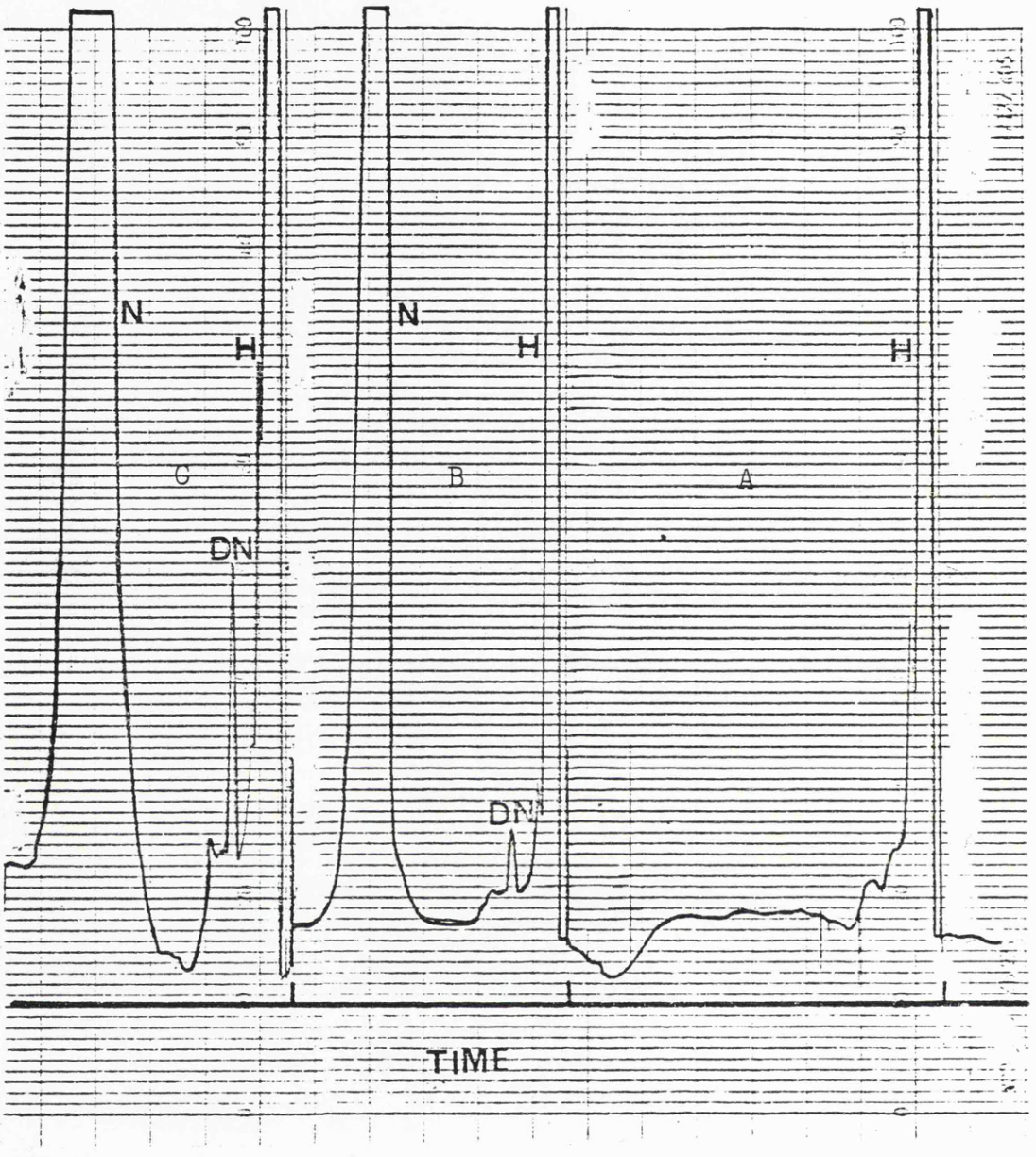
(B) Homogenate incubate with substrate.

(C) Homogenate incubate with substrate and 2mls of β-glucuronidase enzyme.

The incubation reactions were terminated by cooling the flasks in ice and either protein-free supernatant or extracted compounds from the incubate were injected directly onto the column.

The chromatograms on figures 5.14 show typical results obtained using naproxen as the substrate. The chromatographic conditions described in figure 5.10 were

FIGURE 5.14 Homogenate Preparation Containing Naproxen.

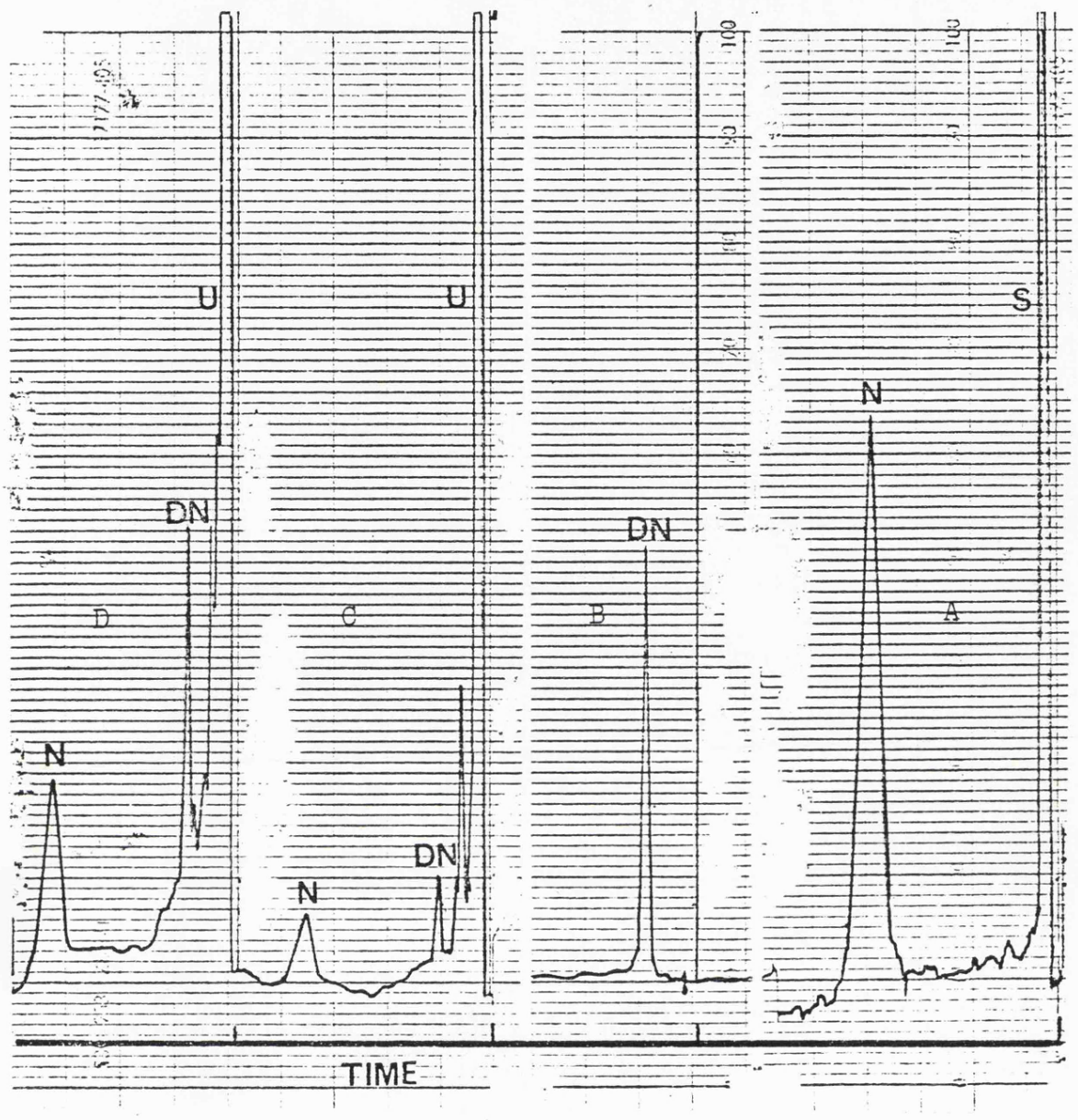


A - Control homogenate injection.
H - Homogenate peak.

B - Injection of homogenate incubate with naproxen
DN - Desmethylnaproxen
N - Naproxen

C - Injection of homogenate incubate with naproxen and β-glucuronidase enzyme.
H - Homogenate peak.
DN - Desmethyl naproxen.
N - Naproxen.

FIGURE 5.15 Clinical Urine Sample Containing Naproxen.



A - Injection of control naproxen
N - Naproxen

B - Injection of control desmethyl naproxen.
DN - Desmethylnaproxen.

C - Injection of clinical urine sample of naproxen.
U - Urine peak.
DN - Desmethylnaproxen.
N - Naproxen.

D - Injection of hydrolysed clinical urine sample of naproxen.
U - Urine peak.
DN - Desmethylnaproxen.
N - Naproxen.

employed. It is interesting to observe the similarities between the result obtained with the liver homogenate and that from clinical urine sample, figure 5.15. It shows:-

(i) Increase in naproxen and desmethylnaproxen after hydrolysis, therefore indicating both are present as hydrolysable conjugates.

(ii) The same pattern occurs in clinical urine and in animal liver homogenate, supporting the general view that conjugation occurs in the liver before excretion of the conjugated glucuronides by the kidneys.

(iii)It illustrates the usefulness of employing animal liver homogenates to study metabolism of drugs.

(iv) Neither the homogenate, nor clinical urine contained sufficiently high levels of metabolites to enable a semi-preparative separation to be worthwhile, indicating the need for dosing healthy animals.

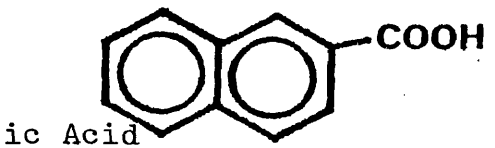
5.3 The Analysis of Drugs and Their Glucuronide Conjugates in Biological Fluids.

Most of the anti-inflammatory agents studies here undergo conjugation with glucuronic acid to be excreted as the glucuronide. Apart from the technique of radioimmunoassay, the glucuronides have not received much attention analytically. The most common procedure for their estimation is by measuring the difference in the value for the free drug before and after hydrolysis. It was felt that HPLC should permit the separation of glucuronide from the free drug and so make it possible to analyze them directly.

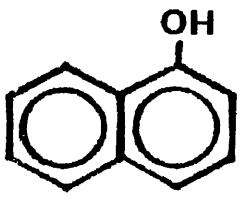
However, no glucuronides of these anti-inflammatory acids were available commercially, or could be synthesized. The most efficient method of conjugating a drug is to use a laboratory animal such as the rabbit, and this was used later, but an analytical technique was first required that could be used to identify the extracted glucuronides.

For this purpose, a range of commercially available glucuronides listed in figure 5.16 and purchased from KOCH-LIGHT Laboratories Ltd., (Buckinghamshire, England), were used to investigate possible HPLC systems for anti-inflammatory drug glucuronides. Reversed phase chromatography using an acidified methanol-water as mobile phase was investigated.

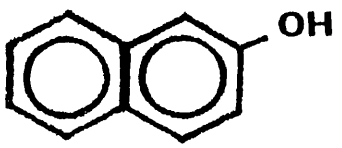
FIGURE 5.16 - Structures of Some Aglycones and Their Glucuronides.



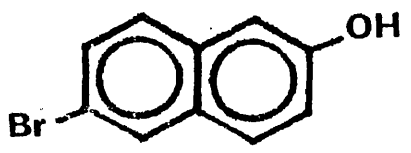
Naphthoic Acid



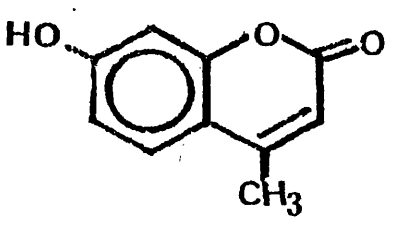
1-Naphthol



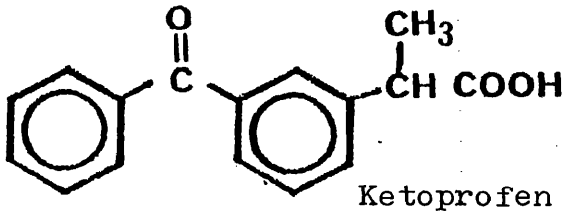
2-Naphthol



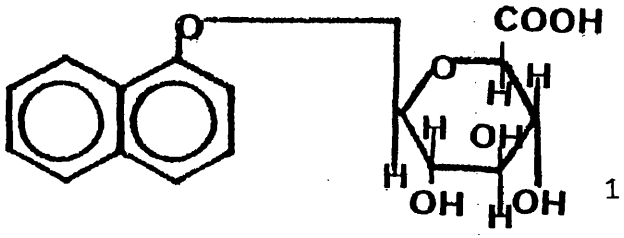
6, Bromo-2-Naphthol



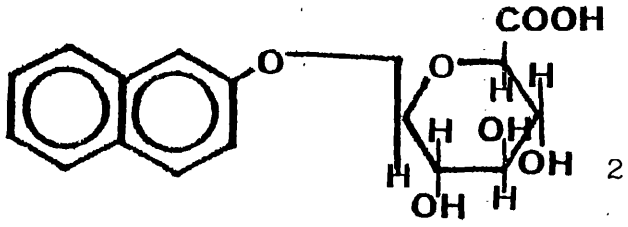
4-Methyl-Umbelliferone



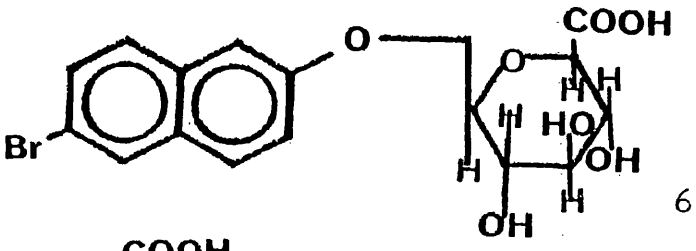
Ketoprofen



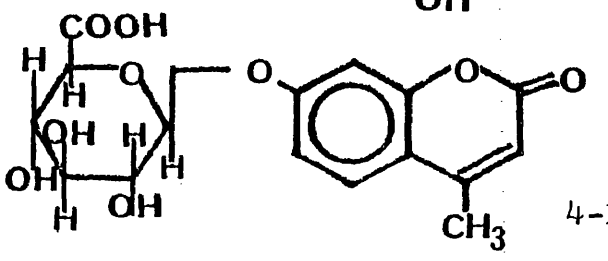
1-Naphthyl-β-Glucuronide



2-Naphthyl-β-Glucuronide



6-Bromo-2-Naphthyl-β-Glucuronide



4-Methyl-Umbelliferyl-β-Glucuronide.

5.3.1 Effect of Eluent Strength on the Capacity Factor (K') and Separation of Aglycones and Their Glucuronides.

Glucuronides are weak acids and they largely account for the excreted forms of most of the arylacetic acids. The chromatography could then be treated as described in Chapter 4 either by suppressing ionization of the glucuronides or by forming an ion-pair with the ionic glucuronides at a higher pH. However, at a higher pH, the glucuronides have been found not to be stable, so that ionic suppression was examined.

At a pH of 2.5, the effect of changing the eluent strength on the capacity factors and selectivity of the compounds and their glucuronides was studied (Table 5.4). Taking a compound and its glucuronide as a pair, the capacity factors were plotted against the % methanol in the mobile phase (figure 5.17).

From these results, it is revealed that:-

(1) The hydrophobic stationary phase (-ODS) was found to be a suitable material since the separation was based upon the relative polarities of the glucuronide and parent compound, with the more polar compound eluting first. It was also an advantage to be able to elute the glucuronide before the parent compound because it ensured the ability to recognise completion of the analysis, especially important when glucuronide standards were not available, thus

TABLE 5.4 Effect of Eluent Strength on Capacity Factor (K′) and Selectivity (α) of the Compounds and Their Glucuronides.

Column - Spherisorb 5-ODS (50x4.6nm id)
Detection - 254nm.
Mobile Phase - Methanol-Water Acetic Acid (pH 2.5)

Compounds	10% MeOH			15% MeOH			20% MeOH			25% MeOH			30% MeOH		
	tR Secs.	K′	α	tR Secs	K′	α	tR Secs	K′	α	tR Secs	K′	α	tR Secs	K′	α
Phenol	63	2.32	-	54	1.84	-	48	1.52	-	43	1.26	-	35	0.84	-
1-Naphthol	555	28.21	2.23	373	18.63	2.79	278	13.63	2.79	194	9.21	2.92	135	6.11	3.14
1-Naphthyl-B-Glucuronide	259	12.63		146	6.68		112	4.90		79	3.16		56	1.95	
2-Naphthol	548	27.85	2.59	349	17.34	2.84	363	12.90	2.95	183	8.63	3.09	128	5.74	3.12
2-Naphthyl-B-Glucuronide	223	10.74		131	5.90		102	4.37		72	2.79		54	1.84	
6,Bromo-2-Naphthol	3504	183.42	3.21	1992	103.84	3.72	1402	72.79	3.66	864	44.47	3.64	600	30.58	2.75
6,Bromo-2-Naphthyl-B-Glucuronide	1104	57.11		549	27.90		397	19.90		251	12.21		230	11.11	
Naphthoic Acid	1730	90.05	-	927	47.79	-	683	34.95	-	431	21.68	-	297	14.63	-
4-Methyl Umbelliferone	399	20	4.42	196	9.32	4.32	152	7.00	4.43	104	4.47	4.47	77	3.05	3.41
4-Methylumbelliferyc B-Gluc.	105	4.53		60	2.16		49	1.58		38	1.00		32	0.90	

Column - Spherisorb-5ODS.
(50x4.5 mm id)
Column Temp. - Ambient.
Detection - 214 nn
Flow Rate - 2ml/min.

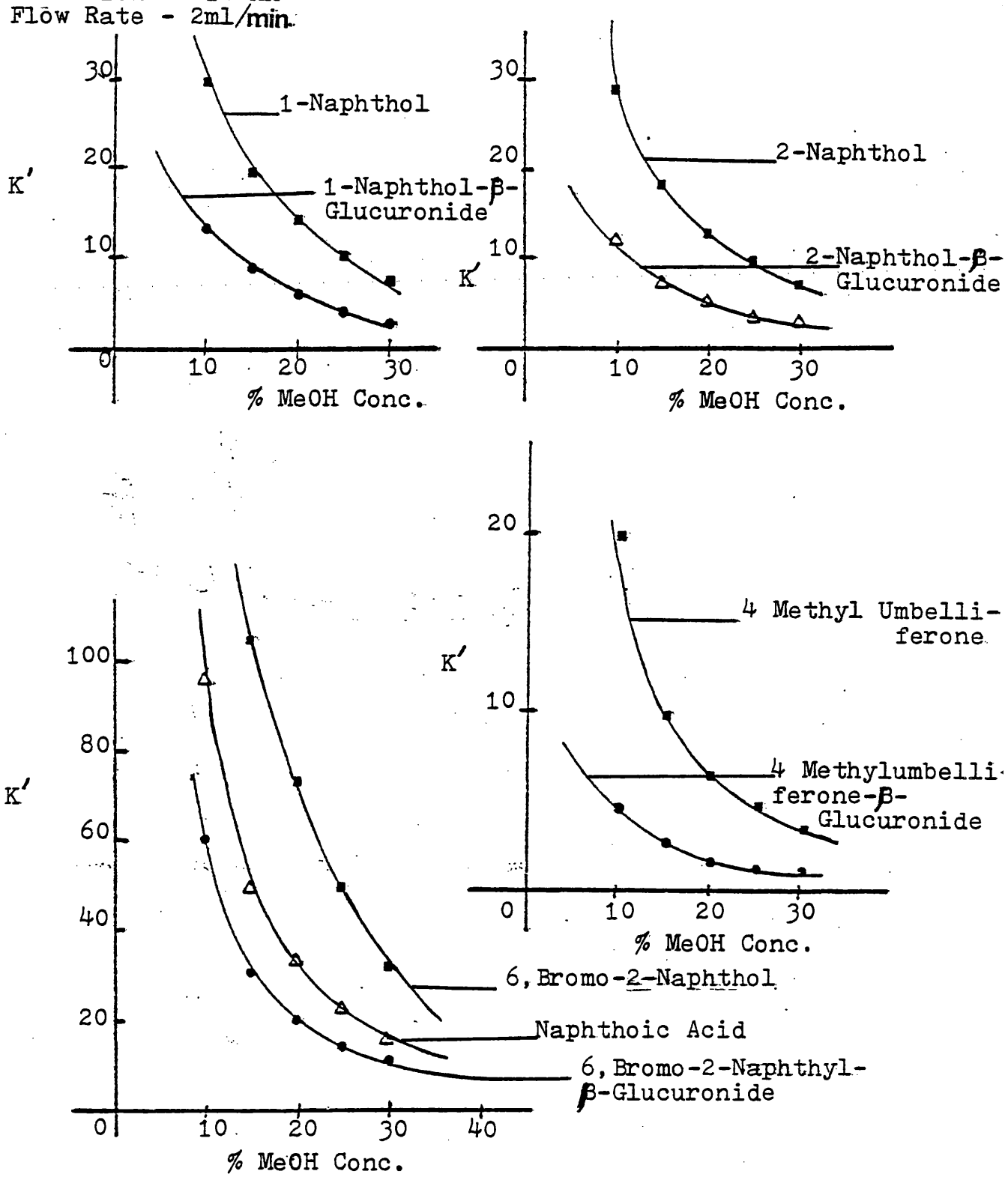


FIGURE 5.17 Effect of Changing Eluent Strength on the Capacity Ratios of Some Commercially Available Compounds and Their Glucuronides.

saving time. In contrast, in normal phase chromatography the glucuronides would elute last and might accumulate on the column thereby gradually degrading the column performance.

(2) The glucuronides were found to be stable at the pH of the mobile phase used, and they gave symmetrical peak shapes. Since most biological fluids were preserved in an acid pH, it was not necessary to adjust or alter the pH before analysis.

(3) Decreasing the % organic modifier was found to increase the retention and capacity factor as well as the separation factor of the glucuronides and the parent compound (figure 5.17). Desired or suitable capacity values for analysis could thus be chosen from the graph - taking separation and analysis time into consideration.

5.3.2 Application.

This procedure could be applied to the carboxylic acids used as non-steroidal anti-inflammatory agents since no analytical method has been reported especially for simultaneous separation and identification of the agent and its glucuronide conjugate.

Large scale preparation of urine rich in glucuronide conjugate was prepared by dosing a healthy female rabbit with 600mg of Orudis capsule (ketoprofen) administered orally. The pooled 24-hour urine sample (about 150mls) was treated in the following ways:-

(1) 1ml diluted to 10mls with the mobile phase and after filtration, 25µl was injected directly onto the column.

Acid hydrolysis of this diluted portion was carried out (figure 4.2) and after filtration 25µl was injected directly onto the column.

(2) An attempt was made to isolate the glucuronide from the urine by subjecting 50mls of urine to basic lead salt precipitation of Kamil, et al. (1951) (70). The yellow gum observed was dissolved in the mobile phase and

(i) 25µl injected onto the column.

(ii) 2mls hydrolysed and 25µl injected onto the column.

(3) The free drug in 50mls of urine was removed by extraction into hexane at pH 2.0. The aqueous layer was then thoroughly shaken with 200mls of ethylacetate. The ethyl acetate was evaporated to dryness at 45°C under vacuum and the brownish yellow gum obtained was dissolved in 10mls of mobile phase.

(a) 5µl from it was injected directly onto the column.

(b) 5µl of the acid hydrolysed portion was also injected onto the column.

In all the 3 cases, similar chromatograms were obtained, figure 5.18, suggesting that G was the glucuronide conjugate while D was the drug ketoprofen, Chromatogram B. After acid hydrolysis, chromatogram C further confirmed that G was the ketoprofen glucuronide and D the drug ketoprofen since the reduction in peak height of G and increase in peak height of D were remarkably noticeable.

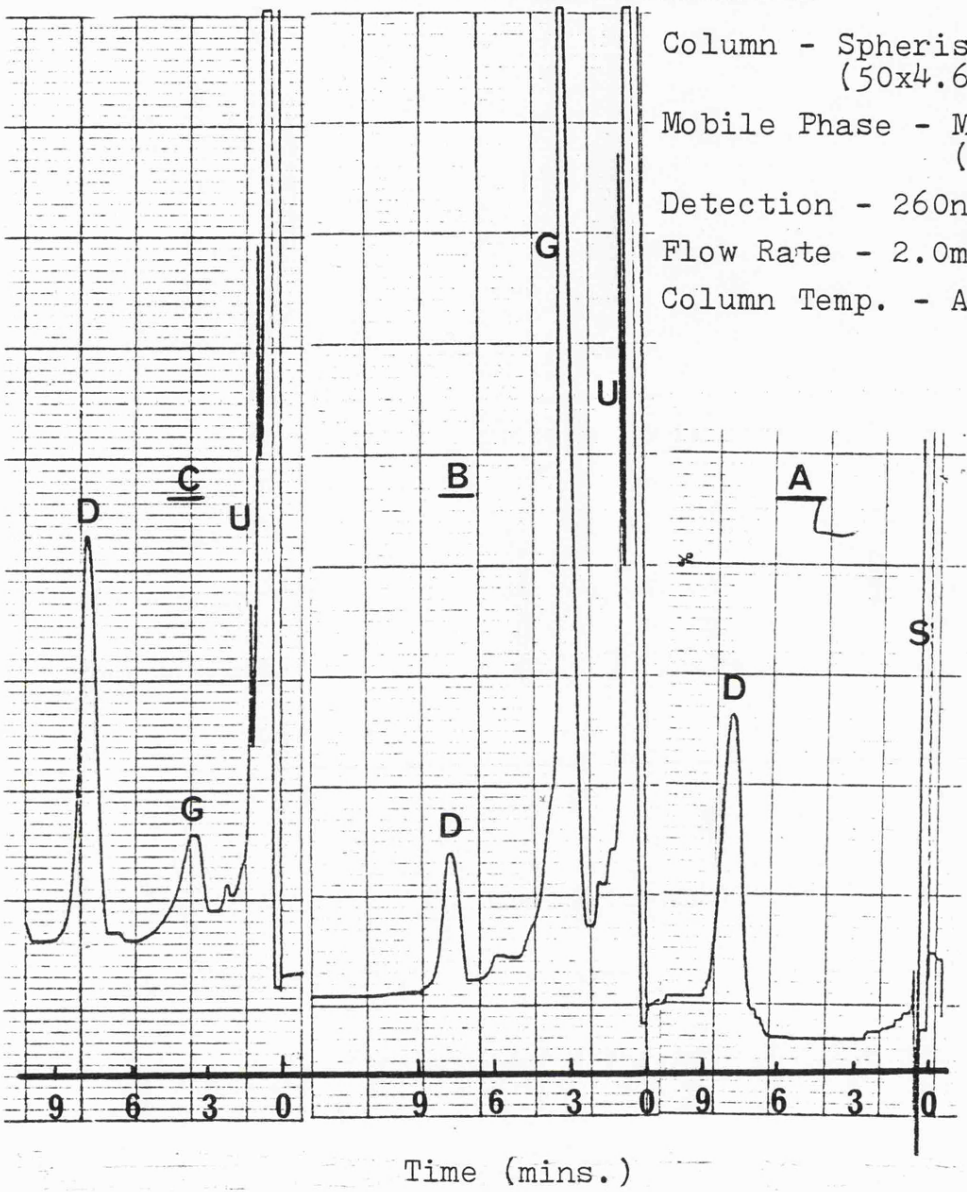
Peak G was collected from the column and hydrolysed and tested for both the aglycone - the drug, by injecting onto the column to produce the peak of the drug. The hydrolysed extract was treated with:-

(i) 0.1% freshly prepared alcoholic solution of carbazole which turned purple (71).

(ii) Fehlings solutions 1 and 2 which turned brown.

These two tests suggested the presence of glucose. Thus peak G is probably ketoprofen glucuronide.

FIGURE 5.18 Separation of Ketoprofen and Its Glucuronide Conjugate in Rabbit's Urine.



A - Control injection of ketoprofen

S - Solvent front.

D - Drug - ketoprofen.

B - Injection of dosed rabbit's urine.

U - Urine front.

G - Ketoprofen glucuronide.

D - Free ketoprofen excreted in urine.

C - Injection of hydrolysed dosed rabbit's urine.

U - Urine front.

G - Ketoprofen glucuronide - (much reduced).

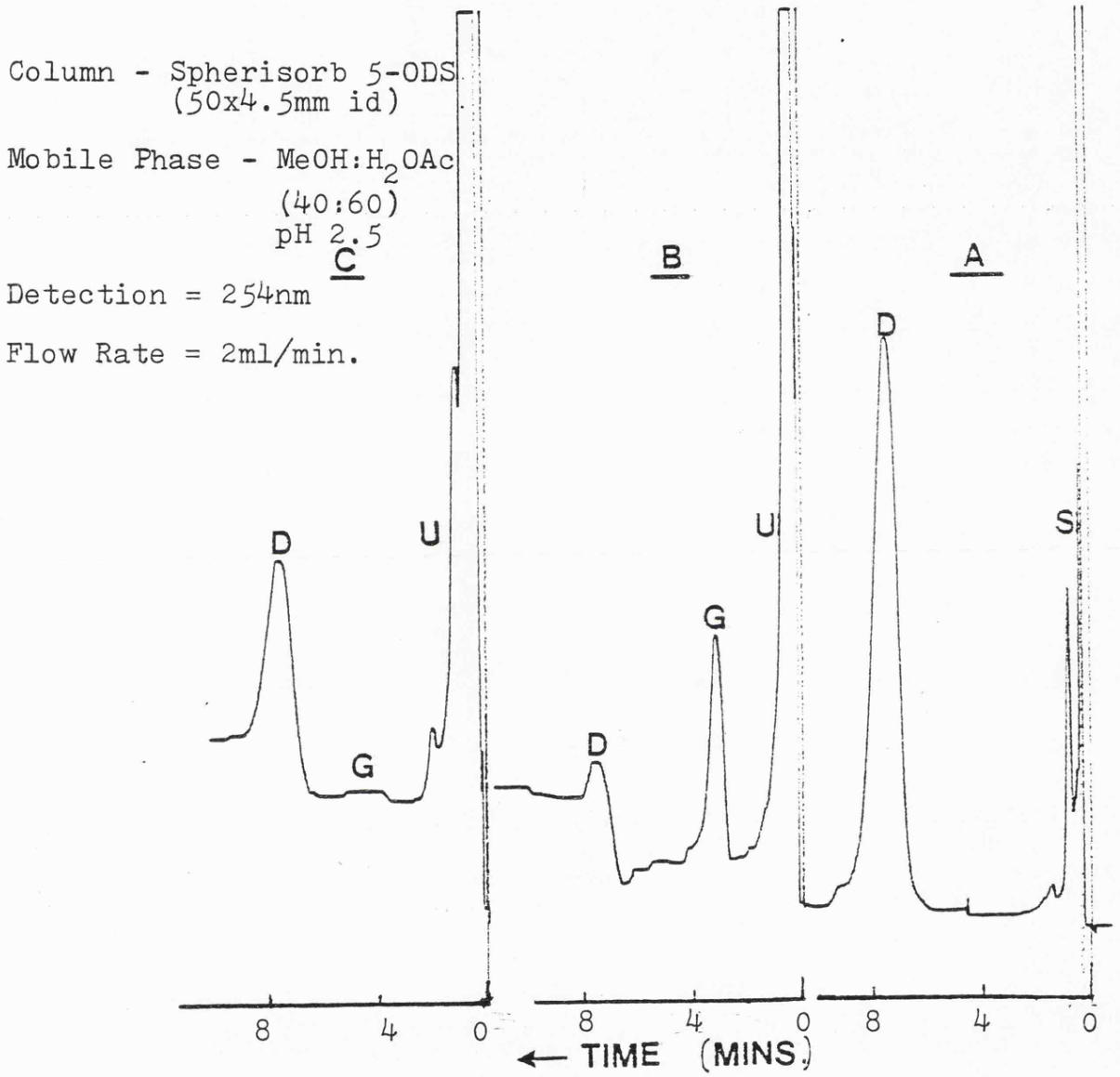
D - Free ketoprofen - (pronounced increase after hydrolysis).

The process was repeated for another drug - benoxaprofen and similar results were obtained (figure 5.19). Chromatograms A, B and C show the benoxaprofen glucuronide and free benoxaprofen.

Analysis of the glucuronide was achieved on a reversed phase column with aqueous acidic methanol by gradually reducing the eluent strength to obtain a satisfactory separation.

Prior extraction or isolation of the glucuronide was not really desirable because of progressive loss of the glucuronide during the process. Attempts to obtain the glucuronide conjugates in crystal form as described by Kamil[70] were not successful. However, concentration of the glucuronide conjugate was achieved by extraction into ethyl acetate evaporation to dryness, and then dissolving in a few mls of the eluent. This method was used by Harman, et al. (1964) (8).

FIGURE 5.19 Separation of Benoxaprofen and Its Glucuronide Conjugate in Rabbit's Urine.



A - Control injection of benoxaprofen sample.

S = Solvent front.

D - Benoxaprofen.

B - Injection of dosed rabbit's urine.

U - Urine front.
G - Benoxaprofen glucuronide.
D - Free benoxaprofen excreted in urine.

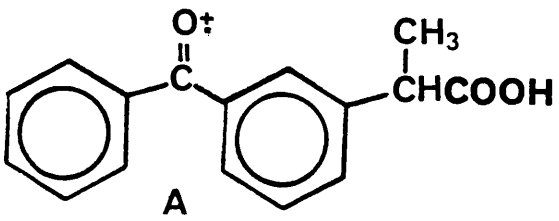
C - Injection of hydrolysed dosed rabbit's urine.

U - Urine front.
G - Benoxaprofen glucuronide - absent.
D - Free benoxaprofen (pronounced increase after hydrolysis).

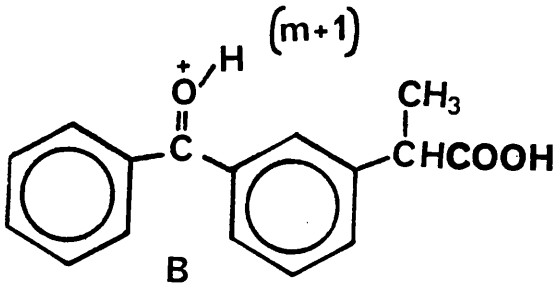
5.4 Field Desorption Mass Spectrometry.

Further attempts were made to confirm the identity of the peaks considered to be the glucuronides of ketoprofen and benoxaprofen. Field Desorption Mass Spectrometry of pure samples of ketoprofen, benoxaprofen and crude extracts obtained from urine considered to contain these glucuronides (figures 5.18 and 5.19) were obtained by the same instrumentation and experimental conditions as described by Parfitt, et al. (1976) (72) for gentamicins.

Spectrum 1, figure 5.20A shows the FD spectrum of a pure sample of ketoprofen (mol.wt. 254) with the parent-ion at m/e254. This could be due to ion A, which occurs in this case instead of the anticipated quasimolecular ion B.

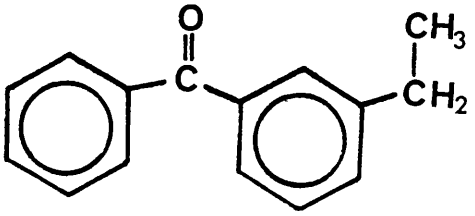


A



B

Decarboxylation of ketoprofen would explain the presence of ion m/e209, i.e. (254-44).



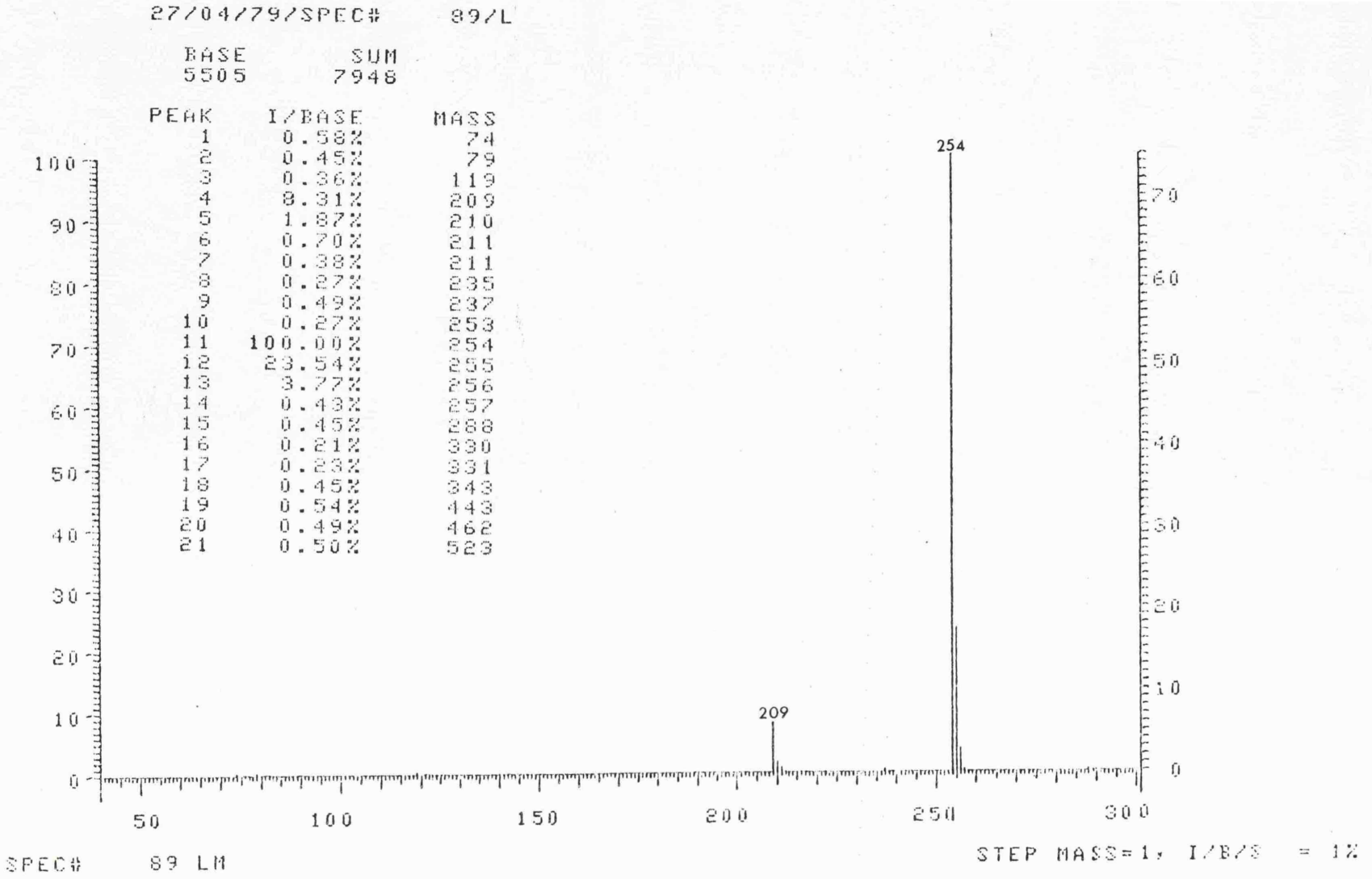
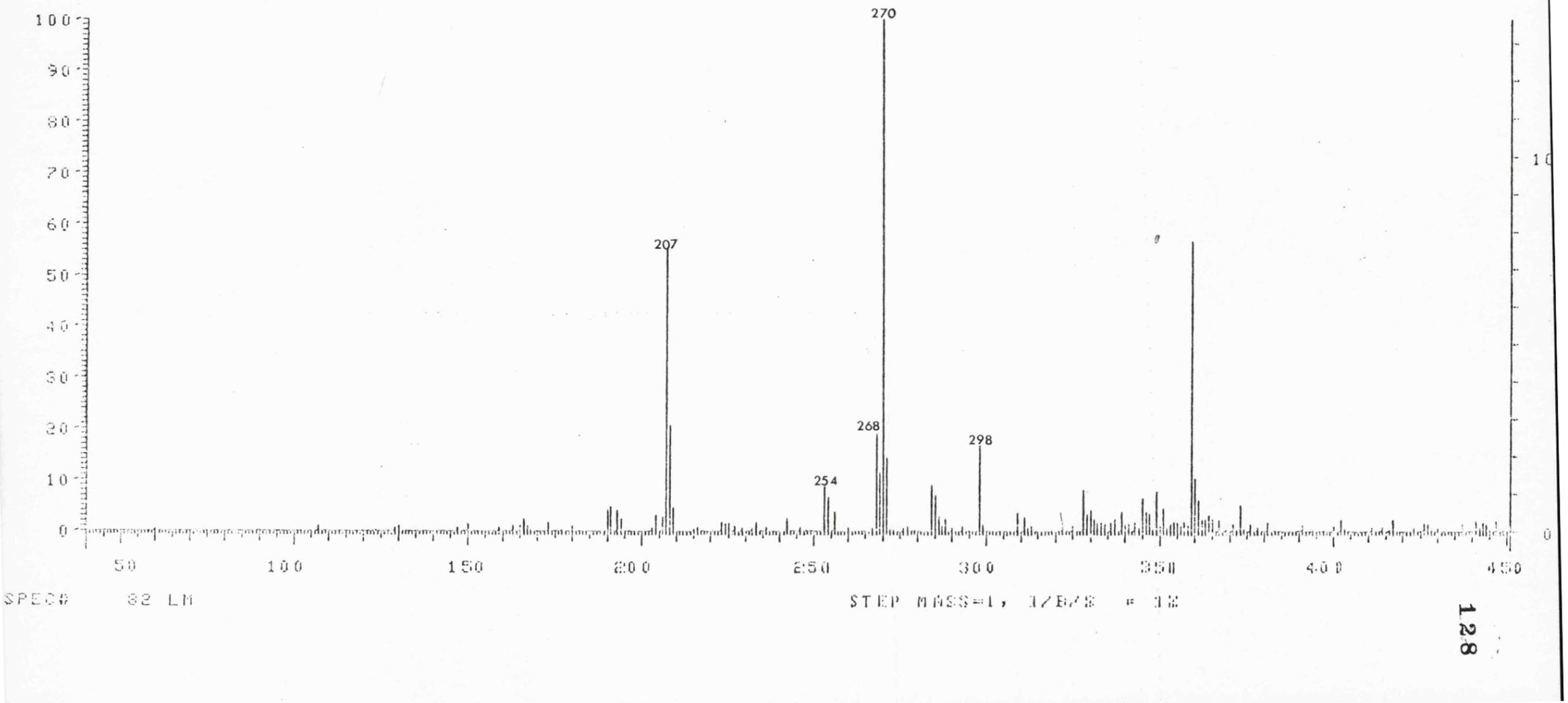


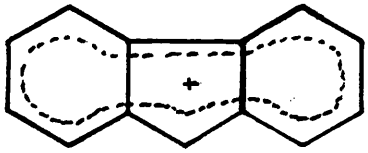
FIGURE 5.20A FD Spectrum of Pure Sample of Ketoprofen.

FIGURE 5.20B FD Spectrum of Urine Extract of Ketoprofen.



Spectrum 2, figure 5.20B, shows the FD spectrum of a crude ethyl acetate extract from the urine of a female rabbit dosed with ketoprofen. Attempts were made to interpret the spectrum as follows.

m/e at 207 - Suggests a very stable ion, probably resulting from ketoprofen (m/e 254) or its proposed metabolite (m/e 270).



m/e 254 - Corresponds to the free drug parent ion.

m/e 268 - Could be ketoprofen, as its methyl ester - (253+ 15 $\longrightarrow$ 268) caused as an artefact by the presence of methanol in the extraction solvents.

The base peak,

m/e 270 - is strongly suspected to be a hydroxy-ketoprofen metabolite since it corresponds to ketoprofen + O quasimolecular ion, i.e. (253+ 17).

m/e 298 - This could then be the ethyl ester of hydroxy-ketoprofen, due to transesterification during extraction.

The presence of hydroxyketoprofen in urine has not been previously reported. The position of the -OH group cannot be located from this spectrum, although NMR (^{1}H and ^{13}C) would offer evidence of the substitution position.

Unlike the crude urine extract analyses on HPLC in figure 5.18, the FD spectrum shows no evidence of a glucuronide of either hydroxyketoprofen P+ m/e 447 or Q+ m/e 448 or of ketoprofen (P+ m/e 431 or Q^{+}m/e432). This could be due to hydrolysis of the glucuronide which could have occurred after extraction and prior to MS analysis, since there was a delay of several weeks.

However, HPLC analysis of a similar extract as shown in figure 5.18 strongly suggests a glucuronide, due to the change in peak heights after hydrolysis. Certainly, HPLC-MS, if available, would be a better technique, particularly under chemical ionization conditions. Spectrum 2 in figure 5.20B could further explain the results in figure 5.18. It is very interesting to note that the acid hydrolysis completely eliminated the peak attributed to benoxaprofen glucuronide (figure 5.19C), but that a significant peak remains for ketoprofen glucuronide, figure 5.18C. Upon further examination of the ketoprofen glucuronide peak before hydrolysis, figure 5.18B, a definite shoulder could be seen, suggesting the presence of a second compound. It is possible that this could be hydroxyketoprofen, which would be unaffected by acid hydrolysis.

These observations emphasise the need for an HPLC-MS analysis of the extract. Meanwhile, FD spectrum of pure sample of benoxaprofen is shown in figure 5.21 with the base peak of parent ion at m/e 301.

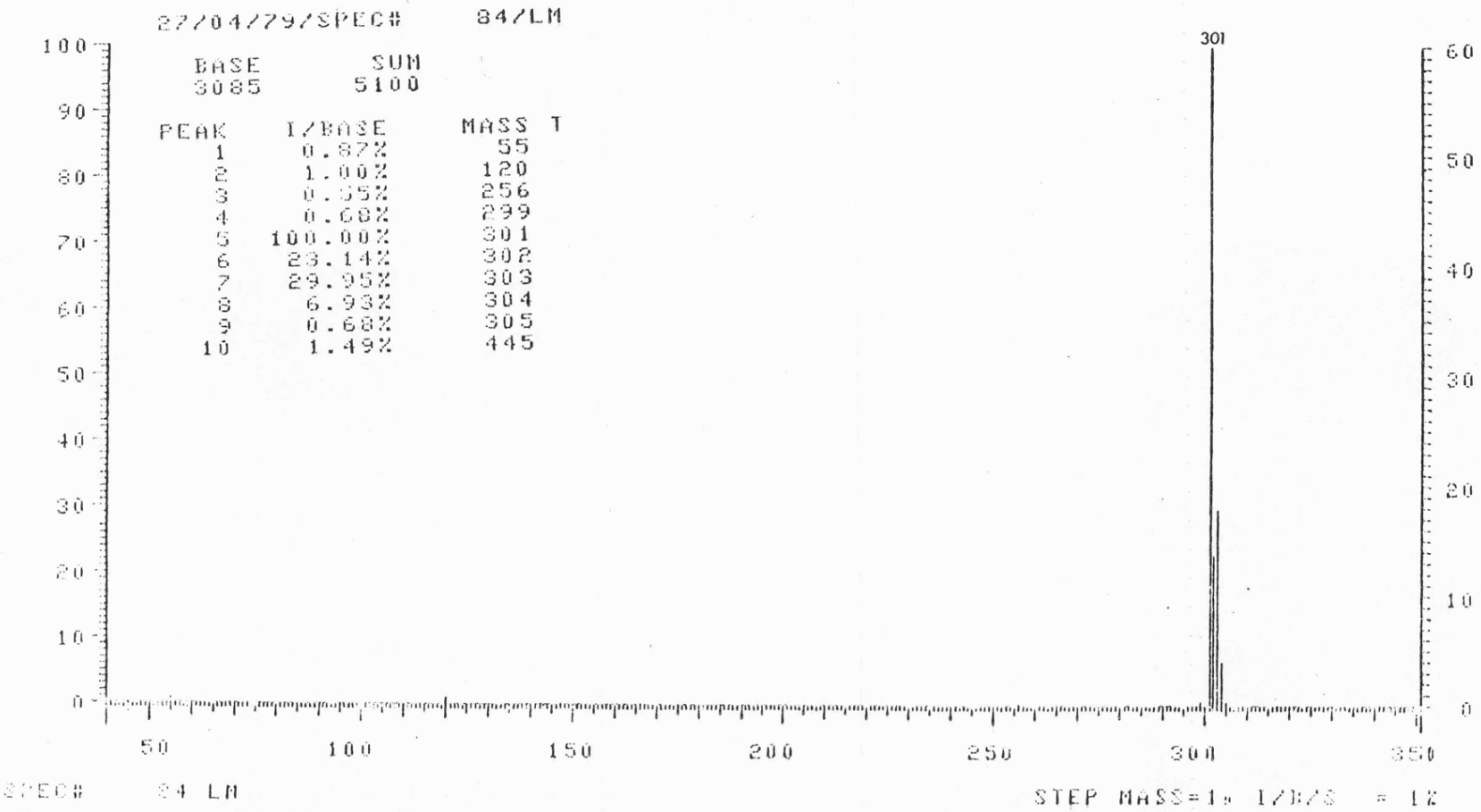


FIGURE 5.21 FD Spectrum of Pure Sample of Benoxaprofen

The role of these new ionisation techniques especially soft ionization in MS is now being fully appreciated in clinical studies especially in drug metabolism where pure standards are not available (73,74). However, combined HPLC-MS (64,75) would be a powerful technique for the identification of a compound or metabolite directly as it elutes from the column.

CHAPTER 6

Conclusion

This study has been a contribution to the analyses of non-steroidal anti-inflammatory agents in body fluids. The recently introduced technique of HPLC has been used to develop simple and rapid analytical methods which would be suitable for the routine examinations of plasma and urine in clinical situations.

Previously reported analytical methods employing spectroscopic techniques (Chapter 1.4) were non-specific and would not discriminate between closely related metabolites. They are less suitable for pharmacological and pharmacokinetic studies because lengthy clean-up procedures are required to minimize background absorption from endogenous materials.

B. Lotti (14) had shown that several analyses can be performed simultaneously using thin-layer or paper chromatography but quantification is difficult. The recently reported gas chromatographic procedures for Alclofenac (75,76), Benoxaprofen (77), Indomethacin (78), and Ketoprofen (79) have afforded a remarkable improvement in sensitivity. Like the previously reported GC methods, [Table 1.2], derivatization is still very important to improve their chromatographic behaviour. The use of mass

spectrometry as a detector in GC-MS (76) has further enhanced the already high specificity of GC. However, this instrumentation is at present too elaborate for routine application. In general, GC methods are not applicable for the analysis of highly polar substances, such as the glucuronide conjugates. There is also a possibility of decarboxylation of some of the non-steroidal anti-inflammatory agents at the working temperatures employed in GC.

The HPLC procedure developed here has advantages over all the previously reported methods in its simplicity and ability to cope with routine analyses of all these agents. Though it is possible for two of these drugs to have the same K' values in HPLC, the wider range of retention mechanisms and operating conditions available makes this less likely than in other chromatographic techniques.

Other advantageous features of this procedure are:-

1. Speed and Convenience: The analysis time obtained for any of these compounds was generally about 5 mins. This was possible because of:-

(i) The use of short columns of 50 to 100mn having good selectivity and efficiency; and

(ii) The sample preparation has been kept to a minimum. Unlike GC or other methods, direct injections of particle free biological fluids were possible. In certain cases where extraction was necessary, simple extraction

procedures as described in figure 5.1 have proved very efficient and reproducible.

The use of an aqueous organic solvent on a reversed phase column permitted rapid mobile phase changes because of rapid re-equilibration. The packing of columns is easier than first thought; the use of toxic organic solvents and derivatizing agents are not necessary. All these points have simplified the procedure to be convenient for routine practices.

2. Sensitivity. By working at a fairly high sensitivity of the detector, usually 0.02 absorbance unit per full scale deflection, (Aufs), and injecting 5µl of the sample of each solute, detection limits between 2.5 and 50ng for all the drugs were achieved, Table 5.1.

The level of the drug detected is a factor of:-

(i) The concentration of solute in the injected solution,

(ii) The coefficient of extinction of the solute in UV, and

(iii) The sensitivity of the detector employed.

The sensitivity obtained by this procedure is comparable with that obtained with GC. However, the sensitivity could still be improved by:-

(i) Detection at the λmax. of the drug,

(ii) Direct injection of biological fluids, thus avoiding any extraction and evaporation procedures which could have caused some loss of the solute.

(iii) Injection of large volumes, 25, 50 or 100µl of the sample. Loss of resolution by injecting up to 50µl or even 100µl is slight, even for short columns, because the injected volume does not expand as in GC.

(iv) Concentration of the compound, only when necessary.

Higher sensitivity could be obtained by derivatizing the compounds for either UV or fluorometric monitors. Jupille (80) and Ahuja (81) have reviewed derivatizing agents for carboxylic acids. These compounds are high dosing drugs and their concentrations in urine and plasma are high enough to be easily detected and quantified without derivatization. Thus derivatizing these drugs was found not only unnecessary but also time consuming and expensive.

The HPLC procedures developed have been used in the analyses of non-steroidal anti-inflammatory agents in body fluids as follows:-

(1) The determination of ten non-steroidal anti-inflammatory agents in plasma and urine (82,83),

(2) This procedure was used frequently to assist clinicians at one Bath hospital to provide rapid evaluations on patients' compliance.

(3) It has also been applied in the clinical trial of benoxaprofen at the Royal National Hospital for

Rheumatic Diseases in Bath (84). This clinical trial was carried out in ten in-patients administered with 600mg of the drug once daily for seven days. Their blood and 24-hour urine samples were collected at the same time for analyses. The range of the drug level in plasma was between 76 and 170ug/ml. This trial demonstrated that the drug possessed both analgesic and anti-inflammatory effects and was well tolerated. The efficacy parameters, articular index and duration of morning stiffness were not well related to the level of the drug in plasma. Non-steroidal anti-inflammatory agents are a group of drugs that do not show a correlation between blood levels and efficacy (85). However, the blood levels of these drugs relate well to the dose, side-effects and also confirm that the drug is being taken by the patient. Further work is in progress in an attempt to relate their blood levels directly with their efficacy, or with other measurable parameters, such as plasma protein sulphydryl levels.

(4) The simultaneous determination of four non-steroidal anti-inflammatory agents and their metabolites in plasma and urine (86).

(5) The profile study of the levels of sulindac and its metabolites in plasma of a healthy volunteer after a single oral dose over 24 hours (Chapter 5.2.2, figure 5.12).

(6) The examination of clinical urine and plasma samples of a patient receiving clinoril and naproxen drugs (Chapter 5.2.2, figure 5.13).

(7) The separation of the glucuronide conjugates of ketoprofen and benoxaprofen (5.3).

The primary objectives of this research have thus been accomplished. The procedure has been recommended to our local hospital, The Royal National Hospital for Rheumatic Diseases, Bath, and will lead to the installation of suitable HPLC instrumentation in the hospital for routine clinical investigations.

This procedure could still be improved by the use of a highly specific detector, mass spectrometry. This will help to simultaneously identify all the separated components in the absence of their standards.

The success of this work has been largely due to the detailed study of the instrumentation of the HPLC technique. This has enabled essential modifications of the apparatus used, especially the coil damping system and useful plumbing connections and by-passes, to be improvised and proved successful. The repairs and maintenance of the instruments have also been carried out during experiments and it is now simple to diagnose, detect and improve faults in the instrumentation by mere examination of the chromatogram. This is an important consideration since this

technique will be further explored for pharmacological, biochemical and clinical studies in a developing country, like Nigeria, where technical assistance, at present, is scarce.

EXPERIMENTAL

CHAPTER 7

Experimental

Details of individual experiments have been included, as appropriate,* to link with the relevant results and discussion in the preceeding chapters.

The following (a) itemises the nature and sources of chemicals and apparatus used, (b) provides a commentary on available HPLC instrumentation, and (c) gives details of chromatographic operating procedures and methods of quantitation used in the present work.

7.1 Nature and Sources of Chemicals and Apparatus Used.

7.1.1 Reagents.

Methanol, acetonitrile, hexane and isopropanol, all HPLC-grade, obtained from Rathburn Chemicals, Walkerburn, Scotland, Ethyl acetate analar grade, sodium hydrogen phosphate, and glacial acetic acid from Fisons Scientific Apparatus, Loughborough, England. Hydrochloric acid (conc) and sulphuric acid (conc) from B.D.H., Poole, England. Cetyltrimethyl ammonium bromide (cetrimide) from Imperial Chemical Industries, Macclesfield, England. β-glucuronidase/arylsulfatase enzyme from Boehringer, Mannheim, Germany.

*Where first experimental detail appears in Results and Discussion, Chapters 4.1, 5.1, 5.2, 5.3.

TABLE 7.1 - Sources of Pure Standards of Drugs Used.

	SUPPLIERS	DRUGS	METABOLITES
1.	BERK PHARMACEUTICALS LTD. Station Rd., Guildford, Surrey, U.K.	ALCLOFENAC (Bx 160505)	—
2.	LILLY RESEARCH CENTRE LTD Windlesham, Surrey, U.K.	1. BENOXAPROFEN (Lot 98A-1207-249A) 2. FENOPROFEN (Lot 45250A)	— —
3.	MAY AND BAKER LTD. Dagenham, England	KETOPROFEN (E.42)	—
4.	M.S.D. RESEARCH LABORATORIES Hoddesdon, Herts. England	1. DIFLUNISAL (Dev Lab No. 5479) 2. INDOMETHACIN (Dev Lab No. 4480) 3. SULINDAC (Dev Lab No. 5279)	— 1. DESMETHYL INDOMETHACIN (L-594, 94700R10) 2. DESCHLORO BENZOYLINDOMETHACIN (L-560,081-00Z07) 1. SULPHIDE (MK 231) 2. SULPHONE (MK 231)
5.	GEIGY PHARMACEUTICALS, Macclesfield, Cheshire England	PHENYLBUTAZONE (Bx 04 5608)	OXYPHENBUTAZONE (MG 1329)
6.	SYNTEX LABS INC. Palo Alto, Calif., U.S.A.	NAPROXEN (Lot B-6-JA-264) (A 7711003)	DESMETHYLNAPROXEN (Lot 3020101) (A 7702015)

7.1.2 Drugs.

Pure standards of drugs used were generously donated by the companies listed in Table 7.1.

7.1.3 HPLC Hardware.

1. HPLC Pumps.

(1) Mini-Pump, Milton Roy Company, Model 396 of Laboratory Data Control, obtained from Phase Separations Ltd.

(2) Jobling HPLC Model JLC 202 fitted with Haskel Dst 122 Pump. Jobling Laboratory Division, Stone, Staffordshire, England.

2. Detector - Variable wavelength - UV Detector, Cecil Instruments CE212. Milton Industrial Estate, Cambridge, England.

3. Recorder: Venture Potentiometric flatbed Servoscribe, Is Re 541.20, Smiths Industries Ltd.

4. Integrator:- Venture Digital Integrator MK11 supplied by Phase Separations Ltd.

5. Injection Valve:- Specac Dual Volume Injection Valve 30.003/30.004 with a fixed operational internal cavity injection of 5µl and interchangeable external loop of 25, 50 or 100µl. Supplier - Spectroscope Accessory Company, Sidcup, Kent, England.

6. Columns - Column tubings, end fittings and plumbing and fitting kits were obtained from HETP, 34 Gonville Avenue, Sutton, Macclesfield, England.

7. Packing Materials.

(1) Reversed phase.

(i) Spherisorb 5-ODS - Phase Separations,

(ii) Hypersil 5-ODS - Jones Chromatography,

(iii) Hypersil 5-SAS - Jones Chromatography.

(2) Bonded silica - normal phase.

(i) Spherisorb $5NH_2$ - Phase Separations,

(ii) Hypersil APS - Jones Chromatography.

8. Solvent delivery system.

This ideally contains solvent reservoirs, pumps, gradient elution device, pressure indicator, flow meter or controller, pulse damper where necessary and in-line filters.

(i) Solvent reservoirs - Glass containers are usually convenient because they are transparent, corrosion proof, inert with respect to the solvent although fragile.

7.2 Commentary on Available HPLC Instrumentation.

7.2.1 Pumps.

There are two types of pumps in common use:-

(i) Constant pressure - Coil

- Pneumatic amplifier

(ii) Constant flow - Reciprocating - Single piston

- Dual piston.

(i) Constant pressure pumps - These consist of some form of pneumatic device for the direct pressurization of

the mobile phase with an inert gas, usually nitrogen. They give a reliable pulse-free flow but the flow-rate and hence elution volumes can vary with changes in permeability of the column or viscosity of the solvent. Examples are:-

(a) Pressurized coil pump - It is the simplest form of pump, consisting of a stainless steel coil filled with the mobile phase andregulated nitrogen pressure used to drive the liquid through the column, figure 7.1A.

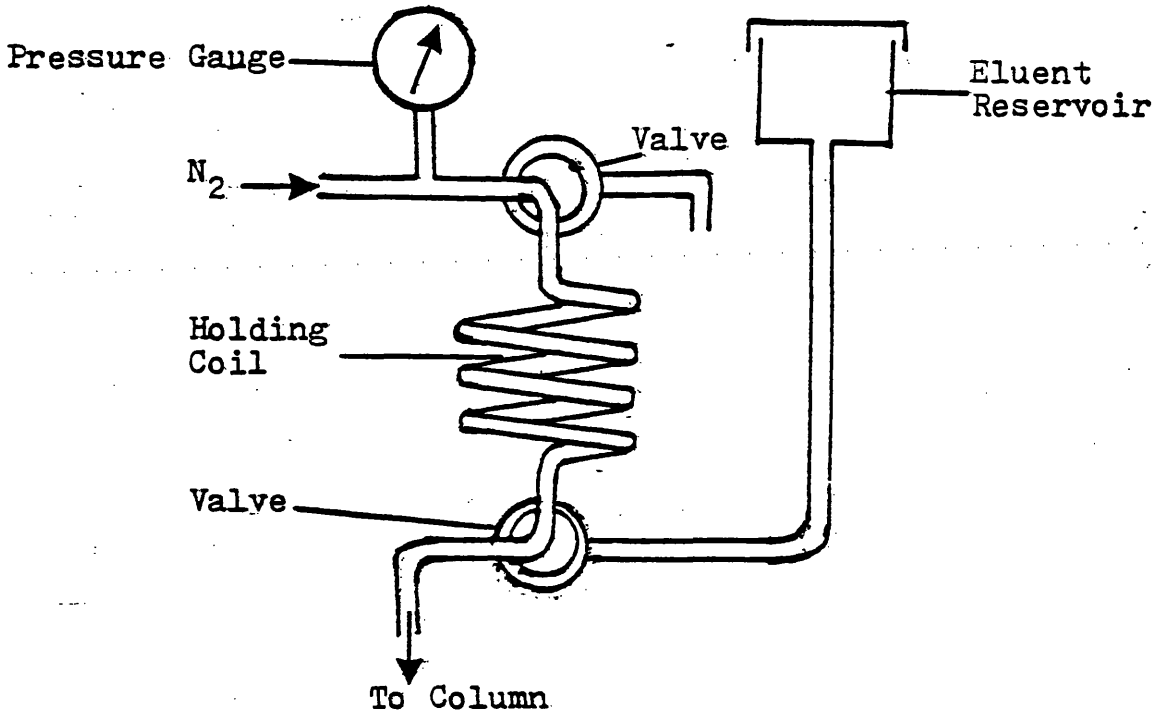
It gives an absolutely pulse-free flow. In practice, it is cheap with minimal maintenance cost, convenient for routine analysis where the same or similar solvent systems often used. But changing solvent is much less convenient.

(b) Pneumatic amplifier - These pumps operate at a constant pressure chosen within a range of 100-5000 psi and the flow rate emerging from the detector is dependent upon the resistance to flow within the entire system. Adjustment of pressure produces the required flow rate which remains pulse free except during the 2 secs required for the automatic refilling step.

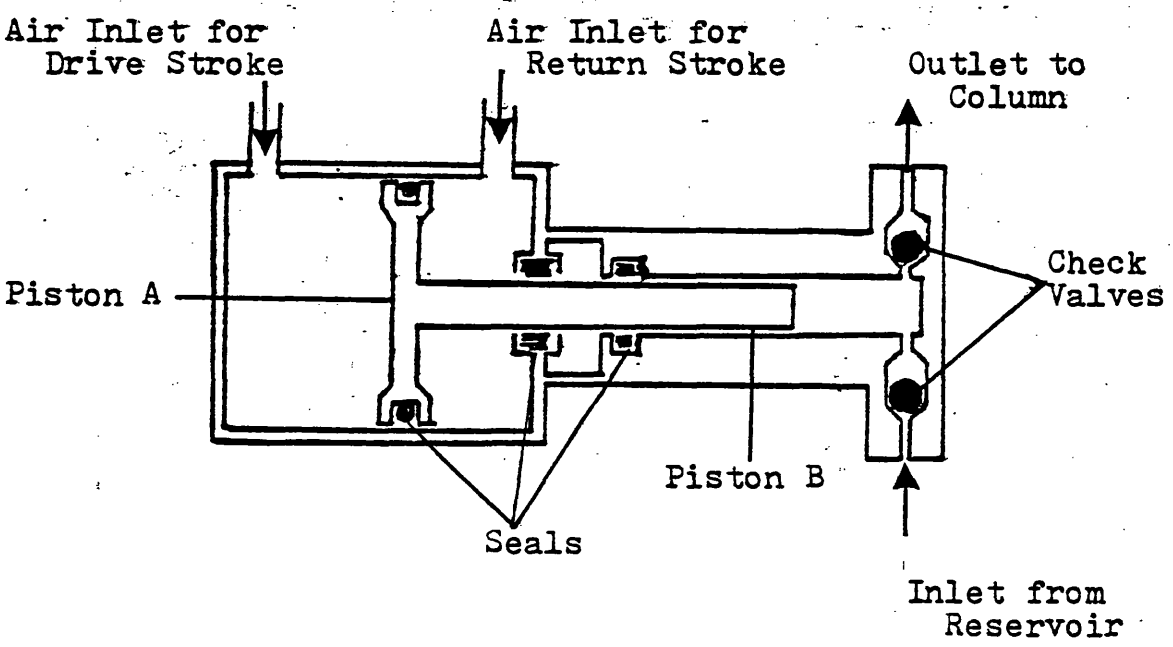
Pressure from an external source, usually nitrogen cylinder, is supplied to a large surface area gas piston attached to a smaller surface area hydraulic piston, figure 7.1B. Thus the applied pressure is amplified and a column pressure of 5000 psi can easily be obtained with a 100 psi gas supply. The gas pressure is released at the

FIGURE 7.1

A. Coil Pump Operated By High-Pressure Gas.



B. Pneumatic Pressure Intensifier (eg. Haskel DST.122)



end of the delivery stroke and the solvent vessel refills by the action of gas pressure or a spring. Check valves between the solvent chambers and the column, prevent the back flow during refilling.

Since they can produce high flow rates (75mls min^{-1}) and high pressures from the moment they are switched on, they are very successful in preparative HPLC and column packing techniques.

Their major disadvantage is the time and volume of solvents wasted when the composition of the mobile phase needs to be altered.

(ii) Constant flow pumps - These are either metering-type pumps, normally mechanical reciprocating devices, or a large syringe-type system. The reciprocating pumps are relatively cheap and incorporated in many commercially available instruments.

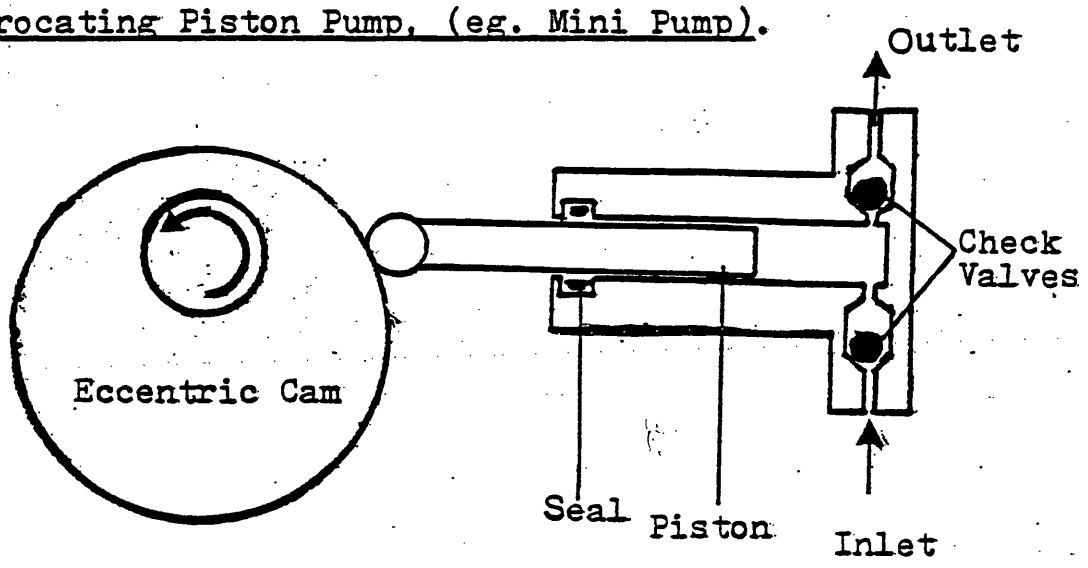
They naturally give constant flow. Small piston types often have constant speed motors synchronized to the supply frequency. Flow rate is altered mechanically by adjusting the piston stroke length; and they can keep pumping as long as liquid is available without a long pause to refill (figure 7.2A).

Disadvantages of these pumps are:-

(a) Limited maximum flow rate makes it less suitable for preparative chromatography.

FIGURE 7.2

A. Reciprocating Piston Pump, (eg. Mini Pump).



B. Dual-Piston Reciprocating Pump Driven By Modified Cardiod Cam, (eg. Constametric I Pump).

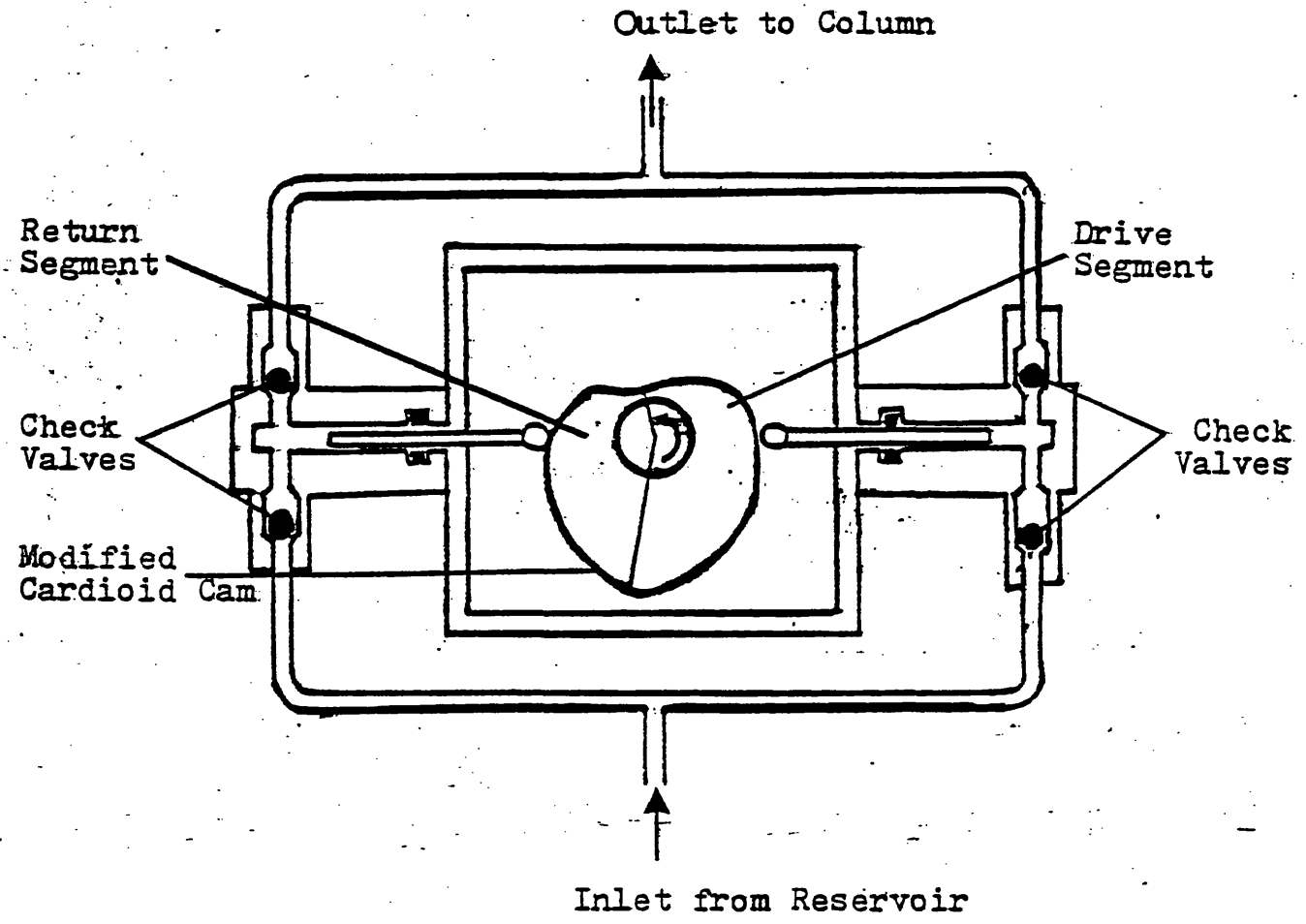


FIGURE 7.2C Schematic of HPLC Set-up Used.

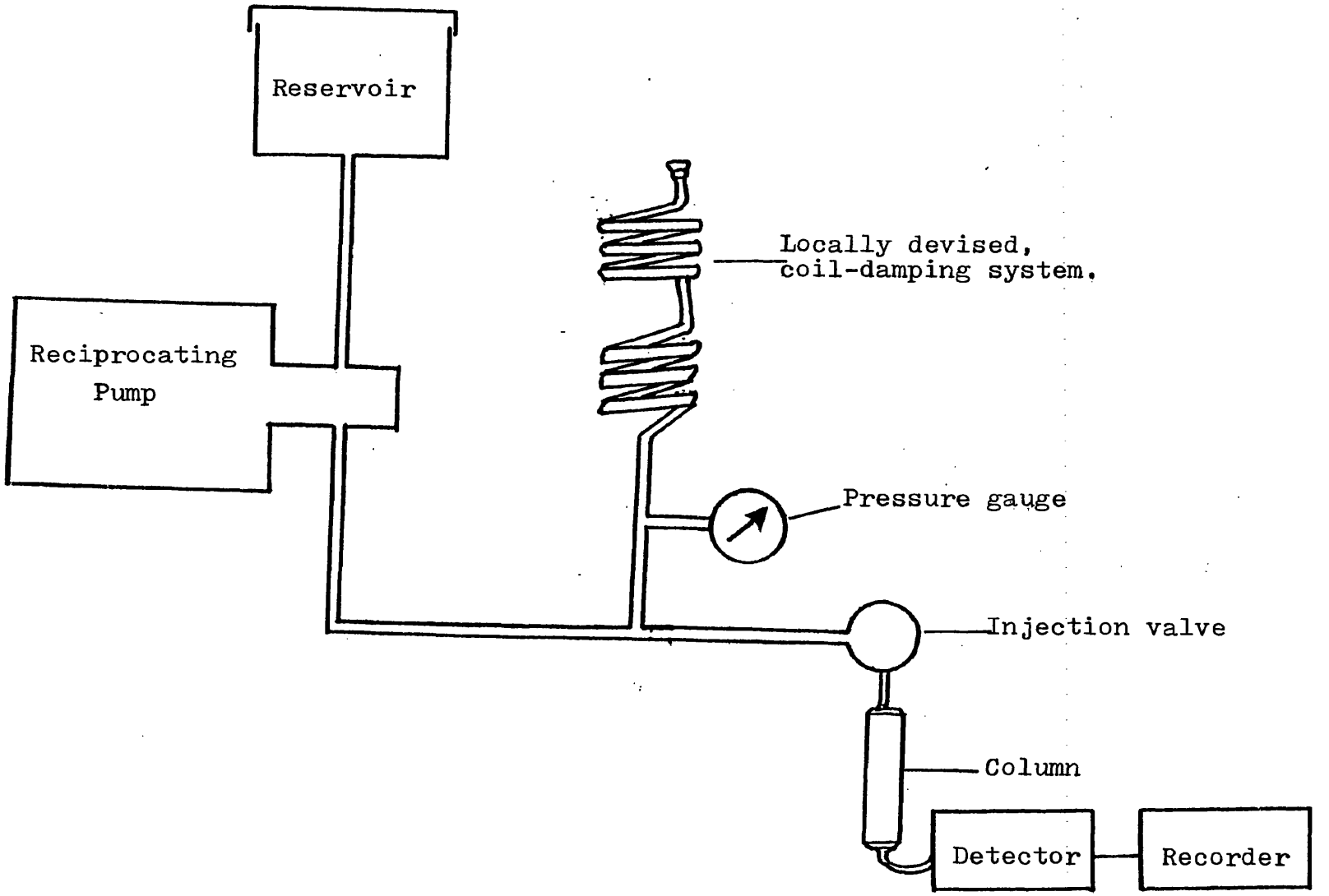


TABLE 7.2 Characteristics of Different Pumping Systems.

Types	Constant	Solvent Change	Convenient for Gradient	Scouting	Cost
Coil	Pressure	Good	Inflexible	Inconvenient	Low
Intensifier	Pressure	Inconvenient	Good for 2 Solvent Systems	Good	Medium
Reciprocating	Flow	Good	Flexible but Inconvenient with Low Pressure Device. Good for 2 Solvent Systems with High Pressure Mixing.	Flexible	Medium to High
Syringe	Flow	Inconvenient	Good for 2 Solvent Systems	Good	High

(b) Pulsating flow which can give rise to considerable base-line instability.

Pulsating flow has been overcome by various devices referred to as 'Damping' which smoothen the flow of solvent delivery to the head of the column by:-

(a) A dual or triple-piston pump (figure 7.2B);

(b) A spring-like tube containing 10ml of air, and introduced daily before pressurization with the pump. This was used during these studies (figure 7.2C).

(c) A flexible bellows or tube, and

(d) A restrictor.

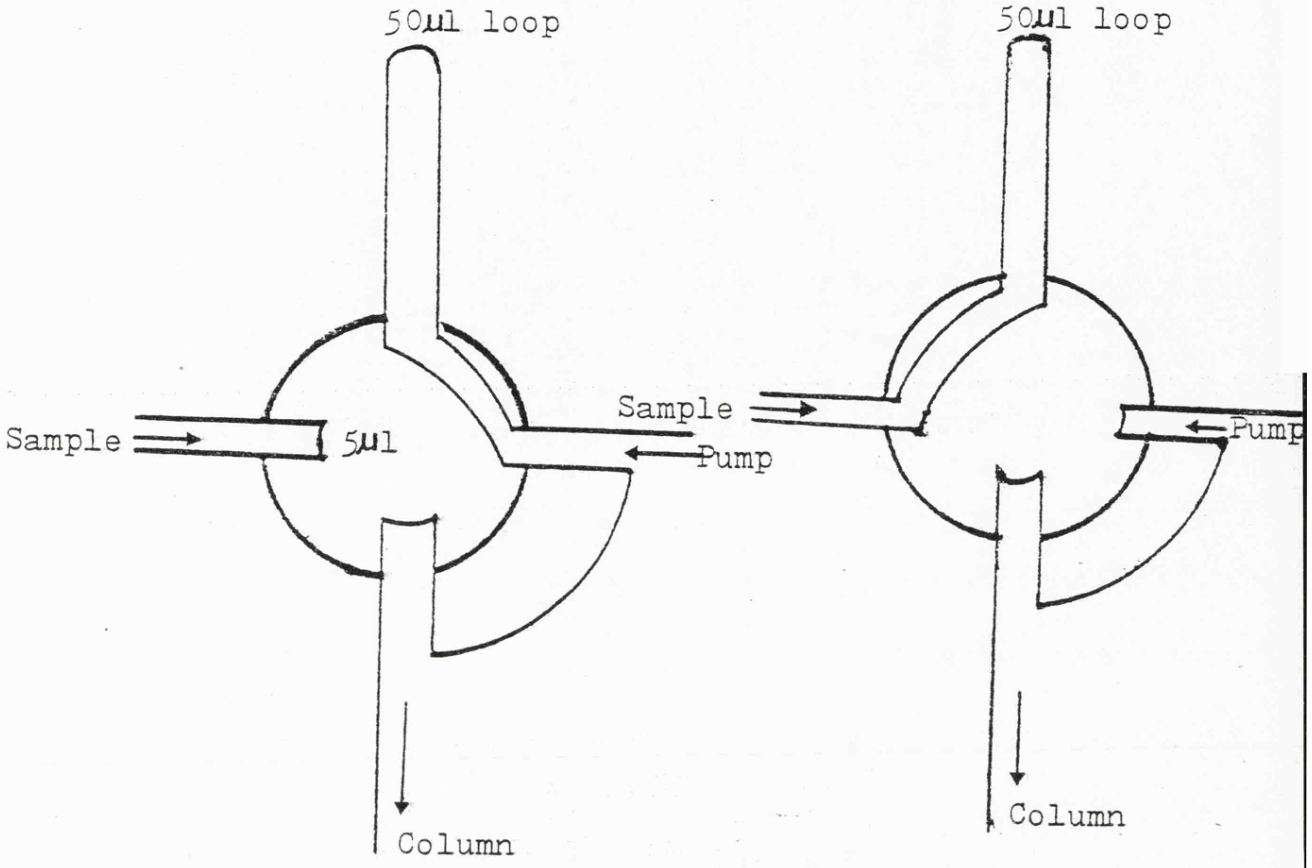
Table 7.2 compares the characteristics of different pumping systems.

7.2.2 Sample injection valve.

Valve injections are capable of introducing very precise volumes of the sample into the chromatographic system. A fixed volume loop is filled with the sample dissolved in the mobile phase, and by turning the shaft, the loop is placed into the solvent stream in front of the column (figure 7.3A).

Sample valves are very convenient and reproducible because injection occurs at the column pressures and all the sample is swept onto the column by the mobile phase. Sample sizes can be varied by changing the sample loop

A



B

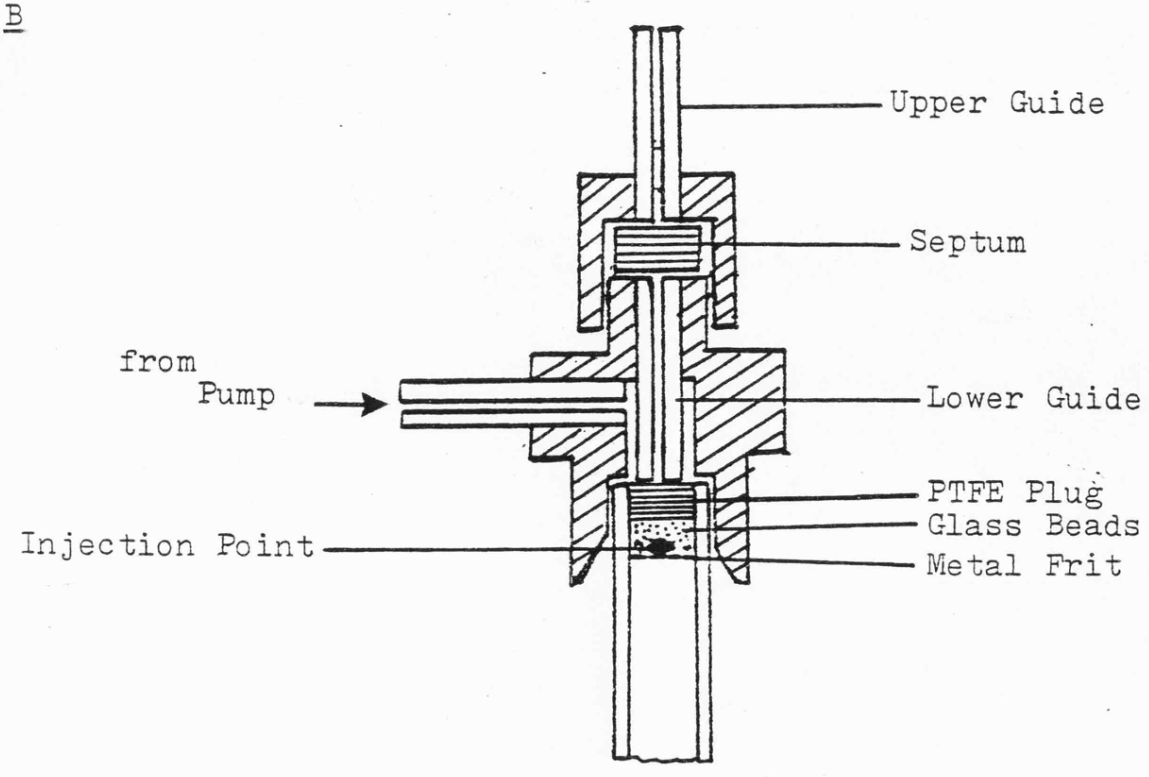


FIGURE 7.3 (A & B) Injection Systems

volume. The injected sample travels some distance in the mobile phase before reaching the column, causing some dilution of the sample by the mobile phase. This reduces the sensitivity, peak shape and efficiency of the column obtained compared with syringe injection. In general, syringe injection produces five times the sensitivity and twice the theoretical plate number compared to valve injection.

7.2.3 Column.

The design, construction and fittings required are discussed under column packing (7.3).

7.2.4 Detection.

Chromatographic detection is a device which indicates the presence of and measures the amount of separated compounds in the column effluent. The two basic types of detectors are:-

(1) Bulk property detectors which measure an overall change in physical property of the mobile phase, eg., refractive index and conductivity techniques.

(2) Solute property detectors which respond to a physical property of the solute which is not exhibited by the pure mobile phase, eg., ultra-violet (UV) light absorption and polarographic techniques.

Important characteristics defining the detector performance are:-

(i) Noise - which is the random signals interfering with the measurement, the frequency of noise compared to the band-width or frequency range of the measurement determines how much noise interferes. Noise limits sensitivity and measurement accuracy, thus minimal noise is required.

(ii) Response. This is the slope of the calibration plot of detector signal against solute concentration or mass rate. Noise, stray light and error in zero transmission setting leads to non-linearity.

UV detectors are commonly used in HPLC because they are selective and frequently have high response. UV monitors with the full-range spectrometers are preferred because they offer greater versatility. They are little affected by environmental changes and so arerather easier to use than many other detectors. Short column-to-detector connection with low dead-volume cells (10 µl) is usually desired since the volume in the flow cells determines the minimum volume that can be sampled but lack of ideal flow through the cell adds markedly to the zone spreading caused by the cell.

The flow-through cell could be adversely affected by gas bubbles issuing from the column which either pass

through, or are trapped in the detector flow cell. This problem is best eliminated at the source by thoroughly degassing the mobile phase before use or by applying a small back-pressure on the outlet of the detector cell by the thumb or by a capillary restrictor.

7.2.5. Recorder.

The subsequent detector signal is amplified and passed to a potentiometric recorder to obtain a permanent record of analysis in the form of a chromatogram.

For this work, both Haskel and Mini-pumps were used to deliver a pulse-free solvent. It was necessary to develop a "home-made" air-coil damping system used with the Mini-pump to obtain a pulse-free solvent flow. The Haskel pump was also used to pack all the columns used in this work.

A variable wavelength UV detector (Cecil Instruments) was used throughout as the monitor and sample injection was by Specac dual volume injection valve already described.

7.3 Column Packing and Testing.

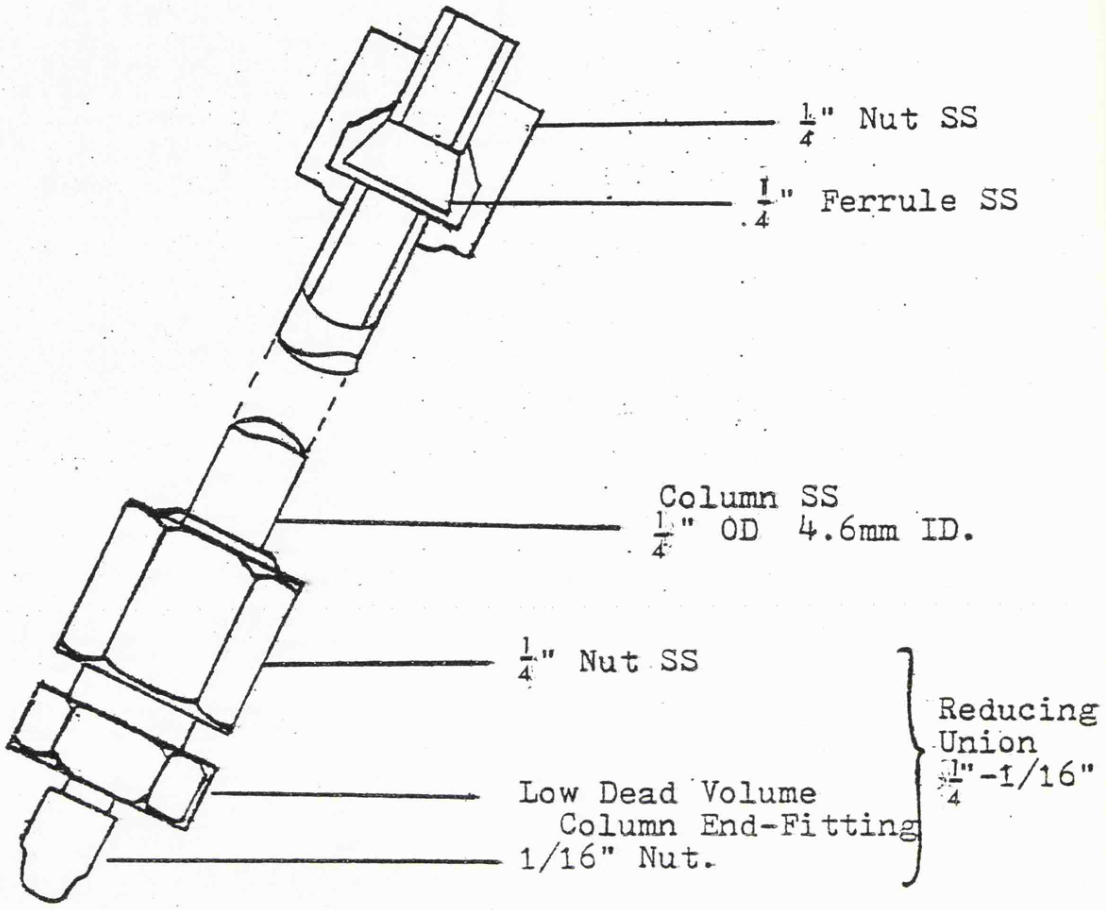
The quality of a column is determined by its efficiency, principally governed by the packing density and the particle size. The main objective of column packing is to reduce band spreading. Prepared columns are commercially available but very expensive. Packing columns allows the free choice of column length to reduce analysis time and is very economical.

7.3.1 Packing Procedure.

The packing equipment that has been used in the diagram, (figure 7.4C,) gave consistently reproducible results with a large number of columns (87,88). All columns used for these studies were prepared from ¼" OD, 4.6mm ID. Stainless steel tubings. An appropriate length of tubing is cut evenly, the ends are smoothed and deburred, and washed with acetone and methanol. A column terminator with a stainless steel frit of 0.5 to 2um porosity was fixed to one end of thetubing, using standard low dead volume connectors, and the column is ready for packing, figure 7.4A.

The dilute slurry technique proposed by Bristow, et al. (1977) (89) was used. Silica, (1.3-1.4gm per 100x4.6 mm ID column) was dispersed by shaking vigorously in 50ml of methanol, and immediately poured into the slurry reservoir, turned upwards. The column was fitted to the

A. Column Design



B. Top (Injection) End of the Column.

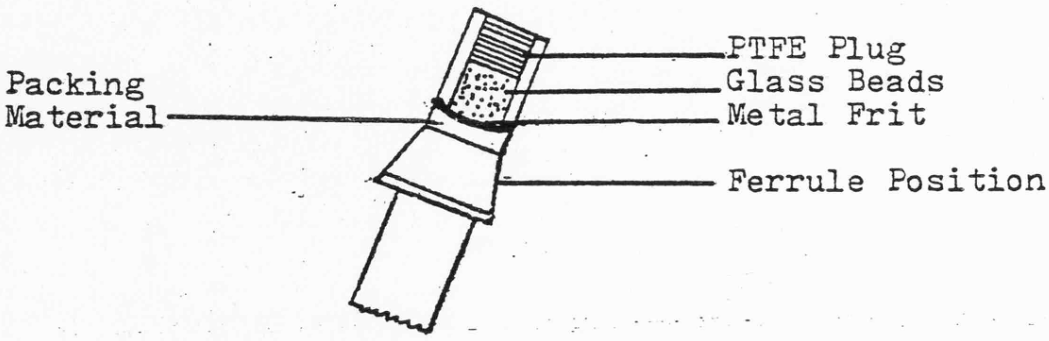


FIGURE 7.4 (A and B)

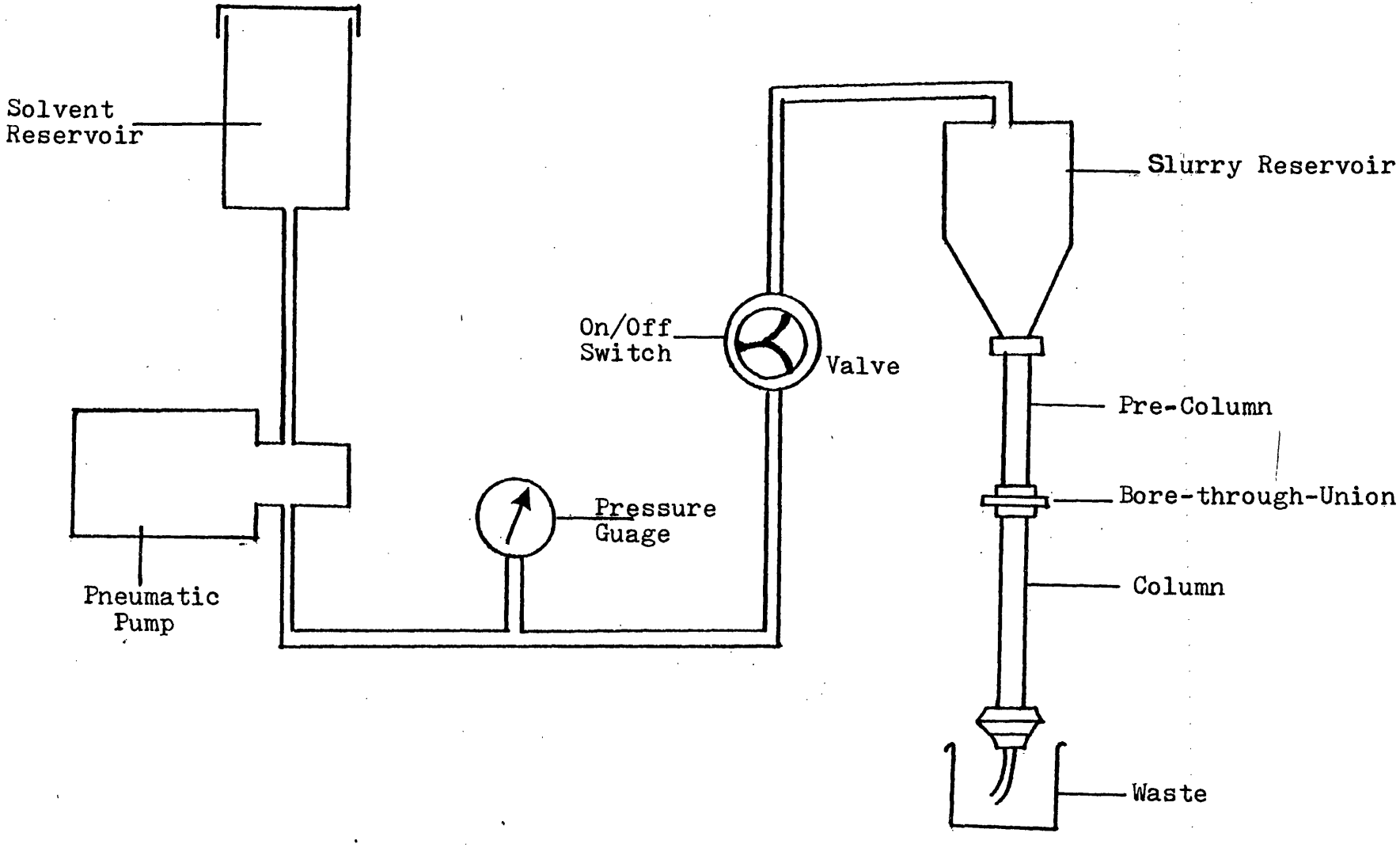


FIGURE 7.4C Slurry Packing System Using a Pneumatic Pump

reservoir and the whole was mounted vertically with the reservoir and the column now inverted, figure 7.4C. The pump was pressurized up to the on/off valve to a liquid pressure of about 4000 psi, and the valve opened. About 250mls of the solvent was passed through after which the pressure was turned off, the solvent flow was allowed to fall to zero and the column left for about 5 minutes before the column was carefully removed.

About 10mm of the packing was removed from the top of the column, terminating it with a suitable metal frit, adding about 5mm of glass ballotini beads (75-150μm) and the column was finally smooth-sealed with a porous PTFE plug. The column is thus ready for use after conditioning with the desired eluent.

7.3.2 Column Testing.

All columns used in this work were tested for their efficiency by an accepted standard procedure proposed by Bristow and Knox (1977) (90). A typical chromatogram is shown in figure 1.15 for a 50mm column packed with Spherisorb, 5-ODS; plate heights (H) of about 0.025mm and reduced plate heights (h) of about 5 were usually obtained.

The same method was used to monitor column efficiency and to indicate any deterioration as a result of injecting biological fluids and large numbers of samples.

Dirty columns were regenerated using pure methanol at a flow rate of 1ml/min for about an hour.

COLUMN EFFICIENCY.

$$N = 5.54 \left[\frac{t_r}{W_{\frac{1}{2}}} \right]^2$$

5-ODS. column
(50x4.6mm.id.)

t_{r_0} – Solvent k′ = 0

t_{r_1} – Phenol k′ = 0.67

t_{r_2} – Anisole k′ = 2.00

t_{r_3} – Phenitole k′ = 3.33

Methanol:Water
60:40 v/v.

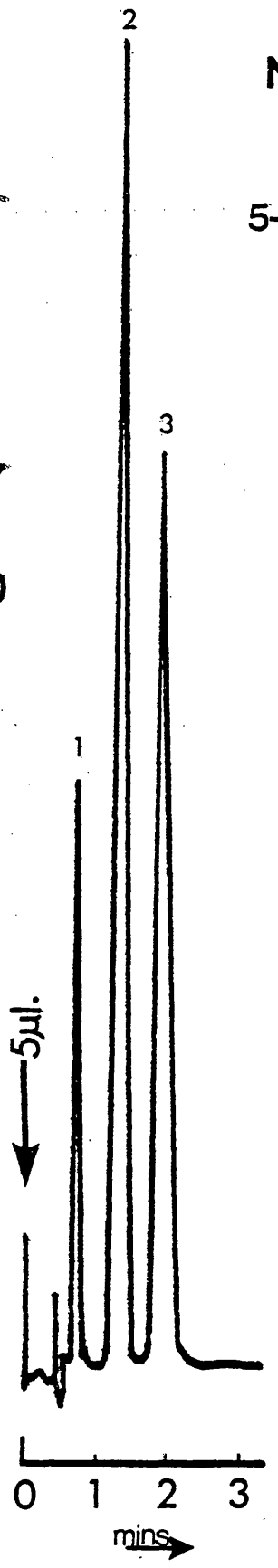


FIGURE 7.5 Column Testing.

7.4 Types of Reversed Phase Columns.

C.M. Riley, et al. (1979) (91) and others (41,47,88, 92) studied the contributory role of commercially available reversed phase stationary phases. The effects of chain length, carbon content, extent of derivatization on wetability, retention and selectivity were examined by them. Three commercially available reversed phase packing materials - Hypersil 5-SAS, Hypersil 5-ODS and Spherisorb 5-ODS, figure 5.2 - were investigated here and further confirmed that:-

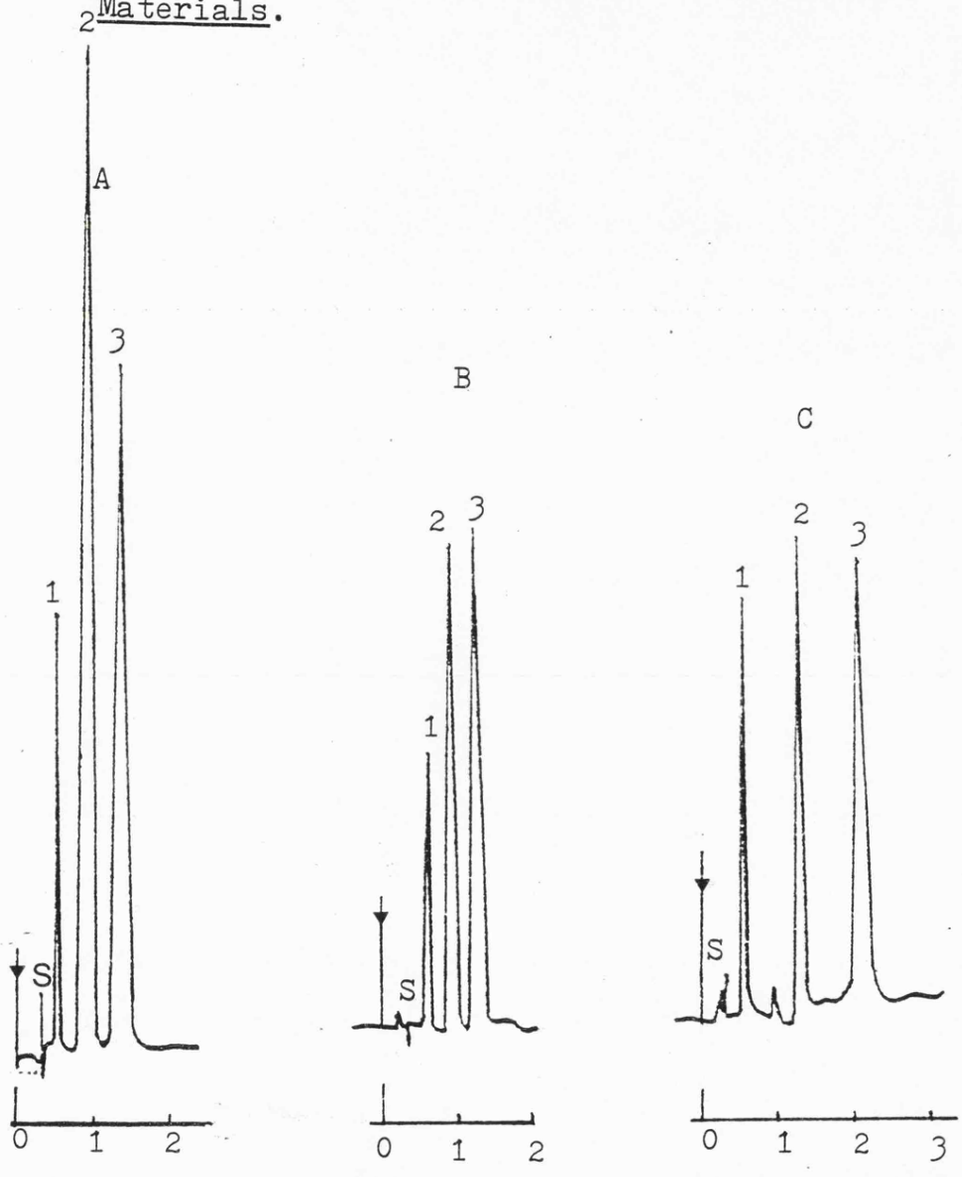
(i) The order of elution of non-steroidal anti-inflammatory agents in all reversed phase systems using the same mobile phase conditions is generally the same.

(ii) Retention and selectivity is increased by increasing the % carbon content bonded to the silica.

(iii) The surface concentration of accessible silanol and siloxane groups also attributes to differences in retention.

Fully silanized accessible OH groups and high carbon content as in Hypersil 5-ODS contribute its higher K' values and better selectivity compared to Spherisorb 5-ODS. The retentive power of the reversed phase stationary phases has been ranked as Partisil ODS-2 > Hypersil ODS> Spherisorb ODS > Spherisorb Hexyl > Partisil ODS, and has been related to the carbon loading of these supports.

FIGURE 7.6 Comparison of Retention in 3 Reversed Phase Materials.



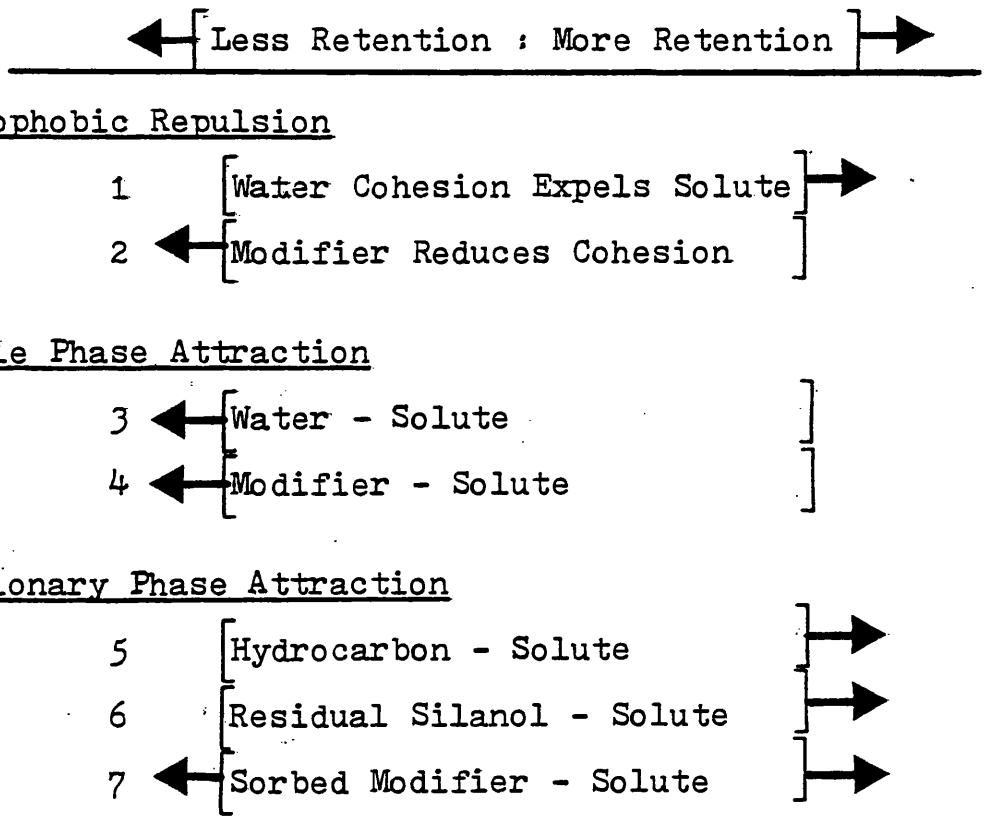
A - Spherisorb 5-ODS 50 x 4.6mm id.
B - Hypersil 5-SAS 50 x 4.6mm id.
C - Hypersil 5-ODS 50 x 4.6mm id.

Mobile Phase - Methanol:Water (50:50)
Detection - 254nm.
Volume Injected - 5µl.
Peaks - S - Solvent
1 - Phenol
2 - Anethole
3 - Phenithole

However, irrespective of the type of reversed phase column used, high selectivity can be achieved by careful selection of the strength of the eluent.

Figure 7.7 illustrates the summary of interactions on reversed phase.

FIGURE 7.7 Summary of Interactions on Reversed Phase.



7.5 Procedures

7.5.1 Preparation of Mobile Phase.

HPLC grade solvents were used for the mobile phase. Each component volume was measured separately before mixing. This ensured reproducibility and accuracy since many solvents when mixed do not give additive volumes due to solvation effects. All mobile phases were freshly prepared to eliminate the possibility of contamination or a change in concentration, especially when buffers and counter-ions were present in the mobile phase.

The prepared mobile phase was filtered using a micropore 0.4μm membrane to remove particles which tend to block the plumbing system, especially the injection valve, then degassed either by refluxing for a few minutes or by passing Helium gas through it for 5 minutes. It is then adjusted to the required pH - usually pH 3 or 2.5 - by titration with glacial acetic acid.

7.5.2 Preparation of Stock Solutions of Standards.

Stock solutions of the drugs used as standards were prepared by accurately weighing 10mg of each sample and dissolving it in 10mls of ethanol to give a strength of 1mg/ml. Suitable concentrations desired were then prepared by further dilutions using the mobile phase.

7.6 Principles of Quantitative Analysis.

Accuracy and a high degree of precision in quantitative HPLC is associated with the following parameters.

1. Injection of the sample. This is achieved by using a syringe or injection valve. It is of paramount importance to introduce the precise volume required directly onto the column. This is difficult but possible using a valve injector, but not generally possible with a syringe. For accurate quantification in the absence of an internal standard it is essential that very accurate injection volumes are obtained.

2. Column and its operational parameters. Column efficiency is very important since it governs the symmetry of the peak. Also, the flow rate must be kept constant since peak area is inversely proportional to flow rate. Pressure fluctuations in the system have an adverse effect on peak area measurements (93,94).

3. Detector response. This is defined as the slope of the calibration plot of the detector signal against the solute concentration. For high precision, the detector response should be linear passing through the origin, i.e. obeying Beer Lambert's Law.

7.6.1 Measurement of Detector Signal.

Once the desired separation has been achieved, the response from the detector representing the mass of solute can be calculated using

(i) Peak height,

(ii) Peak area, by hand planimeter or by electronic integration,

(iii) Cutting and weighing; and

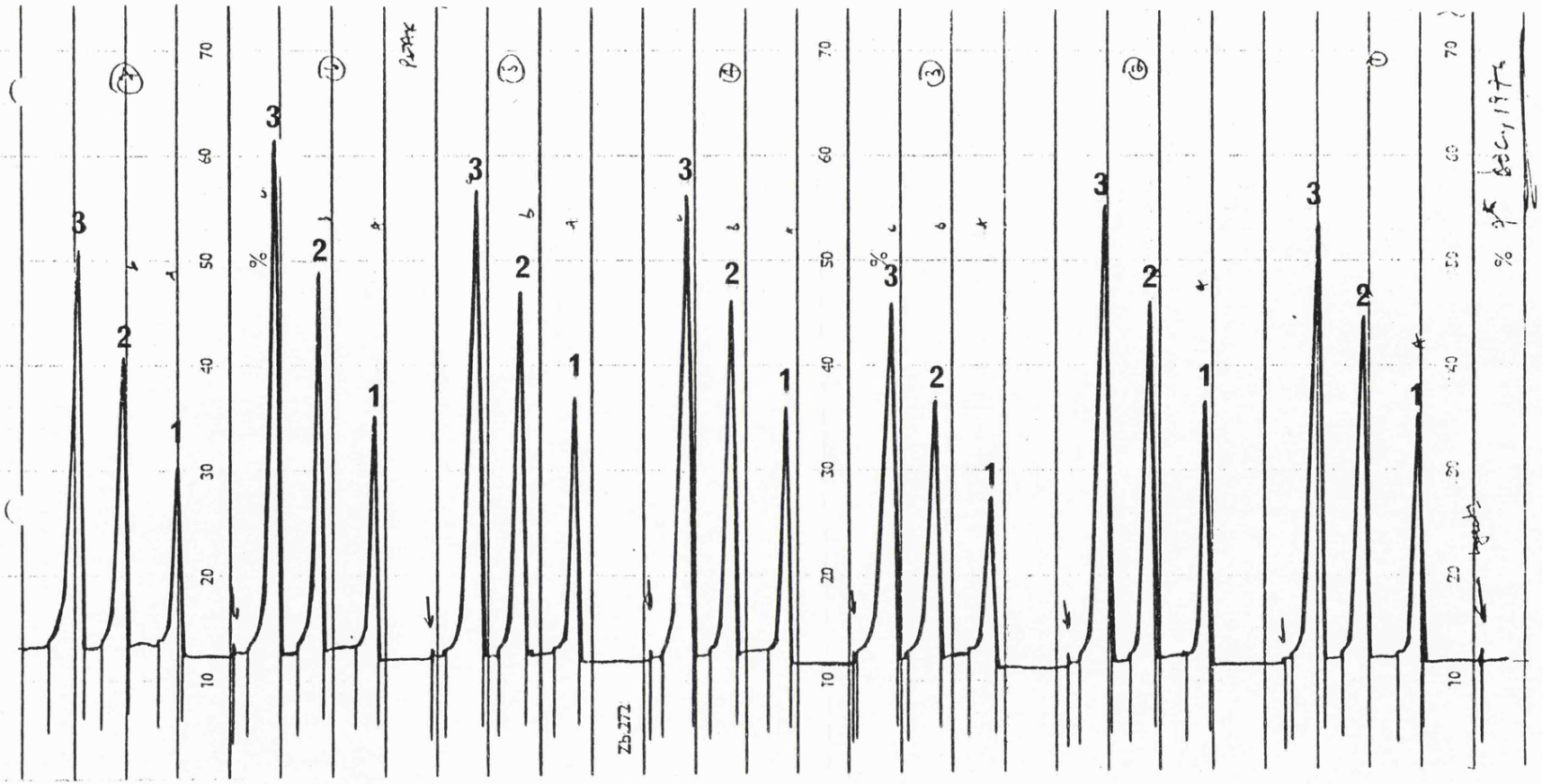
(iv) Triangulation.

From these methods, electronic integration of peak area and peak height measurement have been considered.

The precision of repeated injections of a mixture of phenylbutazone, paracetamol and 2,4-dinitrobenzene (figure 7.8) by comparing the peak height measurement and electronic integration of the peak area were compared (Table 7.3A). In both methods, an internal standard (Table 7.3B) to compensate for minor variations of injected volumes. They both gave a marked reduction in the % coefficient variations from 8% to 3% or less.

Both methods proved to be reliable and suitable for measuring the detector signal with a high precision. The choice of a method was then based on the reliability of the electronic integrator employed and with its cost, peak height measurements could then be considered cheaper, and faster.

FIGURE 7.8 Comparison of the Precision of the Peak Area and Peak Height Measurements.



HPLC INSTRUMENTATION

Column - Spherisorb 5-ODS (200x4.5mm id)
Injection - Valve-5µl Loop
Mobile Phase - Acetonitrile:Aqueous Ammonium Bicarbonate (0.51%)
Ancillary Equipment - Digital Integrator
Drugs - 1-Paracetamol
2-Indomethacin
3-Dinitrobenzene

TABLE 7.3

(A) Measurement of the Peaks in Terms of Peak Height and Peak Area Integration.

Peak No.	Method	No.of Peaks N	Mean Value $\bar{m}$	Std.Dev. $\pm \delta$	%C.V. $\pm \frac{\delta}{\bar{m}} \times 100\%$
1	Height	5	48.400	3.11	6.63
	Area	5	20628	1431.90	6.94
2	Height	5	67.60	2.30	3.41
	Area	5	35548.8	2574.37	7.24
3	Height	5	87.80	5.36	6.10
	Area	5	55873.60	4233.8	7.58

(B) Measurement of the Peaks in Terms of Peak Ratio Using Peak 3 As the Internal Standard.

Peak No.	Method	No.of Peaks N	Mean Value $\bar{m}$	Std.Dev. $\pm \delta$	%C.V. $\pm \frac{\delta}{\bar{m}} \times 100\%$
1	Height	5	0.55	0.01	0.82
	Area	5	0.37	0.01	0.96
2	Height	5	0.77	0.02	3.03
	Area	5	0.64	0.01	0.82

With either method, some basic errors have to be prevented:-

With peak heights:-

(a) Placing of the base line, and

(b) Measuring the height from the base line to the apex of the peak are very significant.

With peak area:-

(a) Placing of the base line in the correct position,

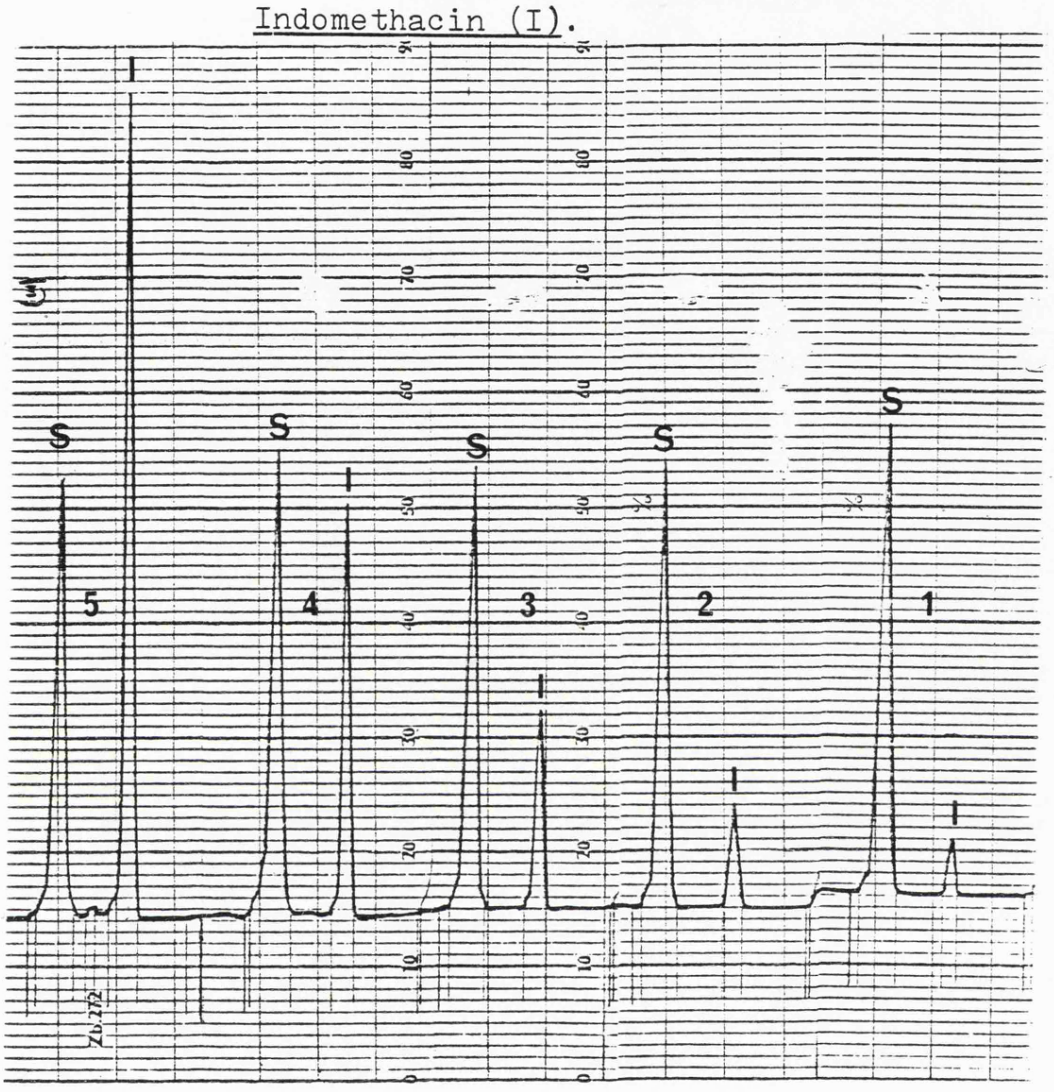
(b) Measurement of the height from the baseline,

(c) Placing of the line and its measurement parallel to the baseline at half peak width, and

(d) The measurement between the sloping sides of the peak.

Calibration plot (figure 7.9A) of the peak height ratio and peak area ratio of indomethacin to the internal standard - 2,4-dinitrobenzene versus the concentration of indomethacin showed the resulting or regression line (figure 7.9B) to be straight and passing through the origin, that is obeying Beer's Law - the intercepts in both cases are not significantly different with zero. Correlation coefficient in both cases are 0.9997 and 0.9998 for peak height ratio and peak area ratio measurements respectively.

FIGURE 7.9A Calibration of Peak Height Ratio and Integral Ratio for Quantification of Indomethacin (I).



CHROMATOGRAPHIC CONDITIONS.

Column:- Spherisorb 5-ODS (200x4.6mm id)

Mobile Phase - Acetonitrile:Ammonium Bicarbonate (0.01%) (70:30)

Detection - 254nm

I - Indomethacin
S - 2,4-Dinitrobenzene (Int. Std.)
1 - 5µg/ml of Indomethacin
2 - 10µg/ml of Indomethacin
3 - 20µg/ml of Indomethacin
4 - 40µg/ml of Indomethacin
5 - 80µg/ml of Indomethacin

Volume Injected - 5µl.

TABLE 7.6

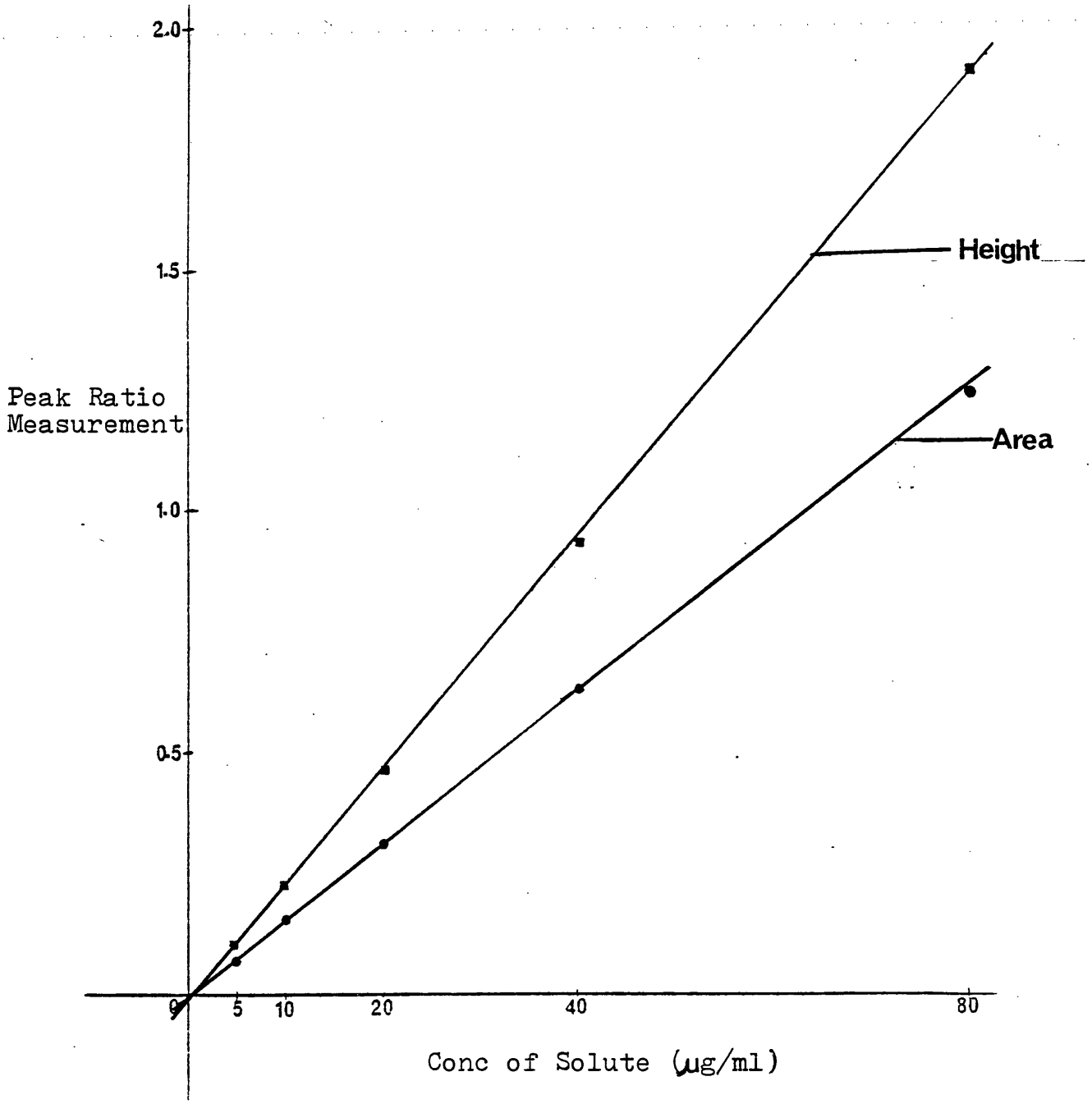
(A) Calibration Plot of Peak Height Ratio and Integral Ratio for Quantification of Indomethacin Using 2,4-Dinitrobenzene as the Internal Standard.

Conc of Indomethacin (µg/ml)		READINGS				
		1	2	3	4	Mean
80	Height	1.82	1.90	2.00	2.03	1.94
	Area	1.30	1.37	1.32	1.33	1.33
40	Height	0.99	0.89	0.90	0.91	0.82
	Area	0.67	0.60	0.62	0.63	0.63
20	Height	0.49	0.46	0.46	0.46	0.46
	Area	0.33	0.31	0.31	0.31	0.32
10	Height	0.23	0.23	0.22	0.23	0.23
	Area	0.15	0.15	0.15	0.15	0.15
5	Height	0.12	0.12	0.12	-	0.12
	Area	0.06	0.07	0.07	-	0.07

(B) Linear Regression Calculations.

	Peak Height Ratio	Peak Area Ratio
Slope	0.02	0.02
Intercept	-0.02	-0.02
Correlation Coefficients	1.00	1.00
When n = 7.5 y =	0.16	0.10
When n = 70.0 y =	1.68	1.15

FIGURE 7.9B Calibration Graph of Peak Height Ratio and Integral Ratio for Quantification of Indomethacin (I) Using 2,4-Dinitrobenzene(D) As the Internal Standard.



Most of the quantification in this work has been based on peak height measurements and have produced low coefficients of variation. It has also allowed minor variations in the flow rate not to interfere with the precision and quantification.

7.6.2 Precautions in Quantitative and Qualitative HPLC.

For:-

1. High Precision of Measurement in Quantitative Analysis, the following precautions were usually taken.

(i) Column flow rate was kept constant.

(ii) Solvent composition was prepared accurately.

(iii) Quantification was made at low noise-level of the detector, and

(iv) Well separated symmetrical peaks were quantified.

Experience has shown more than 50% of the total error of reproducibility was caused by faulty injection technique. Control of column temperature would further improve reproducibility of the separations obtained.

2. Trace Analysis. The elution power of the column and the detection limit of the detector were usually employed to obtain sufficient separation within minimum retention. Thus the need for:-

(i) An efficient column, obtained by choosing a selective packing material, and

(ii) Reducing the retention time by choosing a column as short as possible, eg. 50mm.

APPENDICES.

APPENDIX I

Calculation of Drug Recovery

% Recovery of drug from spiked biological fluid is usually expressed as:-

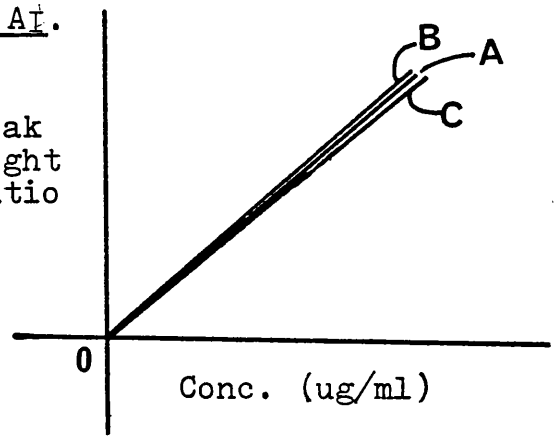
$$\frac{\text{Amount Recovered}}{\text{Amount Spiked}} \times 100\%$$

But for good precision and accuracy, recovery has also been expressed as % recovery over the concentration range of drugs spiked in urine or plasma in the form of a calibration slope.

Typical results obtained from calibrations and recoveries of Ketoprofen from spiked urine and plasma are shown in Table AI, with the chromatogram already on Figure 5.2

With the calibration plots of the recovered drug from plasma and urine, Figure AI would be obtained.

Figure AI.



where A = Drug in mobile phase

B = Drug extracted from plasma

C = Drug extracted from urine.

TABLE AI

```
03 BPRJ10:BPRJ11.LSTSQS,1798 BPRJ10 04 06 11:30:34 12/04/79 022000

03 EOJ 00 4C0251A8 0001     11:31:51

FI TASK 0T    (1798) ENDS

?// TYPE S
REPEAT
?// TYPE DSET99(6100)
FORTRAN IV PROGRAM SHM001 (COMPOSED AS BPRJ11LSTSQS) STARTED 12/04/79 11
        :10:44
EXPERIMENT NUMBER ELUENT
 ****************************

 DATA XTYPE= 0 YTYPE= 0

     X VALUE         Y VALUE         X TYPED         Y TYPED
7260 INVALID CHARACTER.
                  SHM001 TERMINATED ON 12/04/79

CURRENT DATA SET REF. NO. IS 97
CURRENT FORMAT STATEMENT IS:       (8G10.4)(4E1
                                          .(.
               POINTER IS AT CHARACTER     6
RECORD BEING PROCESSED IS:         "PLASMA     5     !     0     0
                                          .(.
          POINTER IS AT CHARACTER     0
CONTROL WAS IN I/0003R
(LEVEL  1)  AT LOCATION 0224DE,= PROG. ORIGIN +000086 = I/0003R+0059C4
ARG. ABS.  PROG. SYMBOLIC
NO.  LOC   ADDRS LOCATION      TYPE          HEX VALUE     VALUE
                        GRAPHICS
 01. 23220 00DC8 SHM001+00DC8    R8 F0F50560F04BF1F3    -6.007897694109009
         D 57             05-0.13

 WAS CALLED BY SHM001, COMPILED 26/07/76 BY VERSION 30 .  STATEMENT CARD
          NUMBER 0011
FORTRAN IV PROGRAM SHM001 (COMPOSED AS BPRJ11LSTSQS) STARTED 12/04/79 11
        :30:50
EXPERIMENT NUMBER ELUENT
 ****************************

 DATA XTYPE= 0 YTYPE= 0

     X VALUE         Y VALUE         X TYPED         Y TYPED
  0.1500000D 02  0.2586000D 01  0.1500000D 02  0.2586000D 01
  0.1000000D 02  0.1712000D 01  0.1000000D 02  0.1712000D 01
  0.5000000D 01  0.8210000D 00  0.5000000D 01  0.8210000D 00
  0.2500000D 01  0.4010000D 00  0.2500000D 01  0.4010000D 00
  0.1250000D 01  0.1840000D 00  0.1250000D 01  0.1840000D 00
SUMX ALL SQ =  0.1139063D 04
SUMY ALL SQ =  0.3283562D 02
SUMXSQ =  0.3578125D 03
SUMYSQ =  0.1048704D 02
SXX =  0.1300000D 03
SXY =  0.2274550D 02
SYY =  0.3979915D 01
SLOPE     =  0.1749654D 00
INTERCEPT= -0.4021635D-01
CORRELATION COEFFICIENT=  0.9999699D 00
STANDARD DEVIATION OF SLOPE     =  0.7638829D-03
STANDARD DEVIATION OF INTERCEPT=  0.8631226D-02
```

```
EXPERIMENT NUMBER PLASMA
 ************************

 DATA XTYPE= 0 YTYPE= 0

     X VALUE          Y VALUE          X TYPED          Y TYPED
  0.15000000D 02  0.25810000D 01  0.15000000D 02  0.25810000D 01
  0.10000000D 02  0.18020000D 01  0.10000000D 02  0.18020000D 01
  0.50000000D 01  0.76900000D 00  0.50000000D 01  0.76900000D 00
  0.25000000D 01  0.36500000D 00  0.25000000D 01  0.36500000D 00
  0.12500000D 01  0.14900000D 00  0.12500000D 01  0.14900000D 00
SUMX ALL SQ =  0.11390630 04
SUMY ALL SQ =  0.32103560 02
SUMXSQ =  0.35781250 03
SUMYSQ =  0.10655550 02
SXX =  0.13000000D 03
SXY =  0.23403250 02
SYY =  0.42348410 01
SLOPE    =  0.18025580 00
INTERCEPT= -0.83526440-01
CORRELATION COEFFICIENT=  0.99871670 00
STANDARD DEVIATION OF SLOPE    =  0.52775030-02
STANDARD DEVIATION OF INTERCEPT=  0.44644840-01


EXPERIMENT NUMBER ELUENT
 ************************

 DATA XTYPE= 0 YTYPE= 0

     X VALUE          Y VALUE          X TYPED          Y TYPED
  0.15000000D 02  0.27090000D 01  0.15000000D 02  0.27090000D 01
  0.10000000D 02  0.13610000D 01  0.10000000D 02  0.13610000D 01
  0.50000000D 01  0.98700000D 00  0.50000000D 01  0.98700000D 00
  0.25000000D 01  0.47770000D 00  0.25000000D 01  0.47770000D 00
  0.12500000D 01  0.23700000D 00  0.12500000D 01  0.23700000D 00
SUMX ALL SQ =  0.11390630 04
SUMY ALL SQ =  0.39334220 02
SUMXSQ =  0.35781250 03
SUMYSQ =  0.12060540 02
SXX =  0.13000000D 03
SXY =  0.23336530 02
SYY =  0.41936930 01
SLOPE    =  0.17951170 00
INTERCEPT=  0.42635820-01
CORRELATION COEFFICIENT=  0.99946130 00
STANDARD DEVIATION OF SLOPE    =  0.34017790-02
STANDARD DEVIATION OF INTERCEPT=  0.23777220-01


EXPERIMENT NUMBER URINE EXT
 ***************************

 DATA XTYPE= 0 YTYPE= 0

     X VALUE          Y VALUE          X TYPED          Y TYPED
  0.15000000D 02  0.27000000D 01  0.15000000D 02  0.27000000D 01
  0.10000000D 02  0.19200000D 01  0.10000000D 02  0.19200000D 01
  0.50000000D 01  0.89000000D 00  0.50000000D 01  0.89000000D 00
  0.25000000D 01  0.50500000D 00  0.25000000D 01  0.50500000D 00
  0.12500000D 01  0.23400000D 00  0.12500000D 01  0.23400000D 00
SUMX ALL SQ =  0.11390630 04
SUMY ALL SQ =  0.39050000D 02
SUMXSQ =  0.35781250 03
SUMYSQ =  0.12079230 02
SXX =  0.13000000D 03
SXY =  0.23524250 02
SYY =  0.42682510 01
SLOPE    =  0.18095580 00
INTERCEPT=  0.26348560-01
CORRELATION COEFFICIENT=  0.99965990 00
STANDARD DEVIATION OF SLOPE    =  0.54141420-02
STANDARD DEVIATION OF INTERCEPT=  0.45800730-01


**FORTRAN ** STOP
***END
?// NOFDEL DSET(S6100)
8202 BPRJ11.DSET(S6100) NOT FOUND.
?// NOFDEL DSET97(6100)
?// NOFDEL DSET99(6100)
?// LOGOUT
 11.48 ETU USED = 01 ETU LEFT = 944757
```

% Recovery over the range of concentration is then expressed as:-

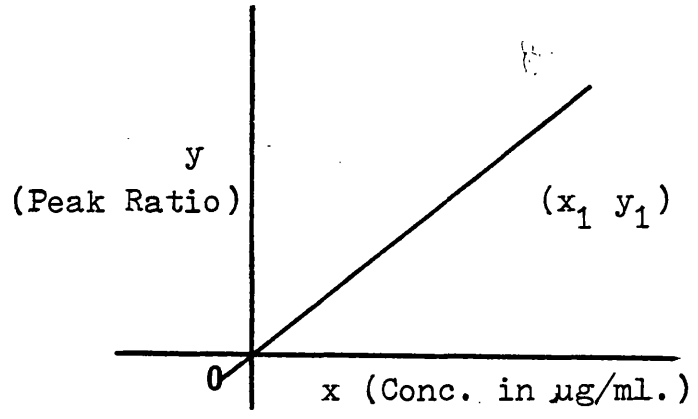
$$\frac{\text{Slope B or C}}{\text{Slope A}} \times 100\%$$

The slopes, intercepts, correlation coefficients, standard deviations of the slopes and intercepts could be obtained from the computer program, Table AI. t-test of the slopes shows no significant difference between them and the recovery is total.

APPENDIX II

Calculation of Linear Regression

All calibration plots were obtained by calculating the linear regression based on the linear equation of the form $y = mx + b$, (Figure A2).



The slope and the y-intercept have been determined as follows:-

$$m = \frac{\left[\frac{\sum x_i \sum y_i}{N}\right] - \sum x_i y_i}{\frac{(\sum x_i)^2}{N} - \sum x_i}$$

$$b = \bar{y} - m\bar{x}$$

where $\bar{x}$ = average x value

$$= \left[\frac{\sum_{i=1}^{N} x_i}{N}\right]$$

$$\bar{y} = \left[\frac{\sum_{i=1}^{N} y_i}{N}\right]$$

σx^2 = Variance of the x values

$$= \frac{\left[\sum_{i=1}^{n} x_i^2\right]}{N} - \overline{x^2}$$

After the linear regression is determined, the degree of association between the random variable (xi,yi) -----(xn,yn), known as the correlation coefficient (r), could be calculated using the expression

$$r = \frac{m\sigma x^2}{\sigma y^2}$$

where σy^2 = Variance of the y values

$$= \frac{\left[\sum_{i=1}^{n} y_i^2\right]}{N} - \overline{y^2}$$

These tedious calculations had been avoided by using a scientific calculator, Texas Instruments, SR-51-II or a computer which automatically computes all the required data, viz:- the slope, intercept, correlation coefficient, mean, standard deviation and variance, Table 7.6.

Typical program obtained for data on Table 7.6 using a computer is shown in Table A2.

TABLE A2

```
?// TYPE DSET99(S6100)
FORTRAN IV PROGRAM SHM001 (COMPOSED AS BPRJ11LSTS0S) STARTED 30/01/79 14
        :37:36
EXPERIMENT NUMBER PEAK HT.GT
 ****************************

 DATA XTYPE= 0 YTYPE= 0

     X VALUE          Y VALUE          X TYPED          Y TYPED
  0.80000000D 02  0.19370000D 01  0.80000000D 02  0.19370000D 01
  0.40000000D 02  0.92200000D 00  0.40000000D 02  0.92200000D 00
  0.20000000D 02  0.46100000D 00  0.20000000D 02  0.46100000D 00
  0.10000000D 02  0.22800000D 00  0.10000000D 02  0.22800000D 00
  0.50000000D 01  0.11800000D 00  0.50000000D 01  0.11800000D 00
SUMX ALL SQ =  0.24025000D 05
SUMY ALL SQ =  0.13439560 02
SUMXSQ =  0.85250000D 04
SUMYSQ =  0.48804820 01
SXX =  0.37200000D 04
SXY =  0.90284000D 02
SYY =  0.21925710 01
SLOPE    =  0.24269890-01
INTERCEPT= -0.19166670-01
CORRELATION COEFFICIENT=  0.99968350 00
STANDARD DEVIATION OF SLOPE    =  0.35264340-03
STANDARD DEVIATION OF INTERCEPT=  0.14561220-01


EXPERIMENT NUMBER PEAK AREA
 ****************************

 DATA XTYPE= 0 YTYPE= 0

     X VALUE          Y VALUE          X TYPED          Y TYPED
  0.80000000D 02  0.13300000D 01  0.80000000D 02  0.13300000D 01
  0.40000000D 02  0.63100000D 00  0.40000000D 02  0.63100000D 00
  0.20000000D 01  0.31700000D 00  0.20000000D 01  0.31700000D 00
  0.10000000D 01  0.14900000D 00  0.10000000D 01  0.14900000D 00
  0.50000000D 01  0.67700000D-01  0.50000000D 01  0.67700000D-01
SUMX ALL SQ =  0.16384000D 05
SUMY ALL SQ =  0.62235280 01
SUMXSQ =  0.80300000D 04
SUMYSQ =  0.22943340 01
SXX =  0.47532000D 04
SXY =  0.68897150 02
SYY =  0.10496290 01
SLOPE    =  0.14494900-01
INTERCEPT=  0.12787040 00
CORRELATION COEFFICIENT=  0.97541750 00
STANDARD DEVIATION OF SLOPE    =  0.18906290-02
STANDARD DEVIATION OF INTERCEPT=  0.75766830-01


**FORTRAN ** STOP
***END
?// NDFDEL DSET97(S6100)
?// NDFDEL DSET99(6100)
?// LOGOUT
 14.59 ETU USED = 00 ETU LEFT = 945466
```

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AND

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